PERTUSSIS VACCINE

SYMPOSIUM

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VOLUME II

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE

NATIONAL INSTITUTES OF HEALTH

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Pertussis Vaccine Symposium--

Division of Biologics Standards

- - -

Conference Room 4A, Building 31
National Institutes of Health
Bethesda, Maryland
Tuesday, October 22, 1963

The meeting was convened at 9:00 a.m., Dr. Grace Eldering acting as chairman in the morning and Dr. R. J. Wilson in the afternoon.

I N D E X

PROCEEDINGS

DR. MURRAY: May we have your attention, please? We would like to proceed with the program. If we could have some quiet, perhaps we could get started.

This morning's program concerns the protective antigens, and we had hoped to have Dr. Verwey here as chairman, but unfortunately he won't be with us, and Dr. Grace Eldering has graciously consented to preside.

So I will turn it over to you, Dr. Eldering.

DR. ELDERING: Thank you, Dr. Murray.

Good morning.

Ever since bacterial vaccines were first used, workers have been searching for ways to isolate the essential immunizing antigens from the rest of the bacterial cell. The first work with the pneumococcus was so encouraging that people thought that the rest of the problem would be simple.

But particularly with pertussis, there have been many attempts over the last thirty years, and people are still working very hard on it. We hope to hear the most recent advances this morning.

The first paper will be by Dr. John Munoz, "Some Properties of Soluble Preparations of B. Pertussis Mouse Protective Antigen."

Dr. Munoz.

DR. MUNOZ: Dr. Eldering, ladies and gentlemen:

A Bordetella pertussis is a complex organism with many antigenic substances of importance to us.

As has been shown with many bacteria, surface antigens seem to be all-important in immunogenicity/ of vaccines.

The surface capsular material demonstrated by some workers -- may I have the first slide, please? -- does not seem to play an important role in establishing immunity in Bordetella pertussis. This capsule may actually be an artifact since it cannot be demonstrated in washed cells by either light or electron microscope.

Second slide, please.

This, by the way, is a picture of electron microscope of washed cells.

The protective antigen is mainly found in cell walls. A picture of the cell walls is shown in the next slide. (Slide 3)

These cell walls can be seen here under the electron microscope. The observation that the protective antigen is concentrated in the cell wall does not imply that intact cell walls are required for protective activity. It seems that only one antigen of the cell walls carries a protective activity.

(Slide 4)

The next slide shows diagrammatically a simplified antigenic picture of B. pertussis. This capsular material which is questionable -- the cell wall and the protoplasm -- the capsule, I have tentatively put the agglutinogen and hemagglutinin as portions of this capsule as though -- as I said, this is not

known whether the agglutinogen is actually a capsule or not. I am not prepared to say. The cell wall has the protective antigen, the histamine sensitizing factor and the endotoxin and the protoplasm has the heat labile toxin and many other antigenic components.

The cell wall, as you can see, is already free of the heat labile toxin, of most of the agglutinogen and hemagglutinin and various other antigens found in the protoplasm.

(Slide 5)

The next slide illustrates the many antigens found in soluble materials from B pertussis. Only one of these antigens has the ability to immunize mice against intracerebral challenge with Bordetella pertussis. The protective antigen can be obtained in solution by various means. Autolysis, sodium desoxycholate or lysozyme under certain conditions releases varying amounts of protective antigen.

In our hands, the most convenient and reproducible method has been that of extracting acetone dry cells with saline at alkaline pH. Briefly, the method is outlined in the next two slides. (Slide 6)

Whole live cells are treated with three volumes of acetone and filtered. The cell paste is again treated with the same volume of acetone and again separated by centrifugation or filtration and the acetone discarded and the cell paste dried.

The next slide. (Slide 7)

This cell paste, dry cells now are suspended. Fifteen

grams of these dried cells are suspended in 500 ml of saline, homogenized and pressurized in a pressure cell of ~~Reeby~~ Ribi and diluted a little more by adding 500 ml more of saline. The pH is then adjusted ~~at~~ to 8.5, incubated overnight at 2 to 5 degrees, centrifuged at 27,000 g's for 40 minutes, and the supernatant results as a saline extract. The saline extract material is the one I will be talking about this morning.

The sediment after this first extraction can be re-extracted two more times with 200 to 400 cc's of saline, centrifuged, and the supernatants added to this material. And you get considerably more protective activity.

The composition of the material which we call "saline extract" is shown in the next slide. (Slide 8)

It contains 14.6 per cent nitrogen, 4.5 hexose, four-tenths hexosamine, 1.8 phosphorus and 28.4 fatty acid esters plus fatty acid amides.

This material can be kept indefinitely in a lyophilized form. Its activity ranges from 10 to 20 gamma per mouse.

In other words, this amount will protect mice from intracerebral challenge with B. pertussis.

These saline extracts are still impure and contain perhaps as many as 12 different antigenic materials. They can be further purified by means of starch electrophoresis where a protective fraction containing mainly one antigenic material is obtained.

Unfortunately, the starch releases a considerable amount of contaminating materials. And for this reason, the exact chemical nature of the protective antigen has not yet been established.

Saline extract forms a clear, but opalescent solution in saline or phosphate buffers at pH 7 or above. It does not go in solution readily at lower pH's. Its activity does not deteriorate very rapidly in solution or in the frozen state. And as I mentioned before, it is very stable under lyophilized conditions.

Formalin deteriorates the protective activity quite readily while merthiolate does not as shown in the next slide.

(Slide 9)

This slide shows the effect of formalin and merthiolate on mouse protective activity of saline extract incubated at a concentration indicated in this slide of 37 degrees for a week.

This (indicating) is the formalin, this is the merthiolate. Formalin at .5 per cent, merthiolate at 1 to 10,000.

As you can see, the protective activity is very nice in merthiolate at 20, 40 or 80 gamma while with the formalin, it requires 80 gamma to show the protective activity.

Similar observations have been previously made, of course, by Dr. Pittman and others with whole cells. If the concentration of the saline extract is increased, the deterioration takes place at a slower rate as shown in the next slide.

(Slide 10)

This is exactly the same experiment with a concentration of saline extract increased to 1.6. As you can see, the deterioration can be shown at 10 gamma, but not at 40 or 160. Merthiolate shows full activity.

The protective activity for saline extract is heat labile as can be seen in the next slide. (Slide 11)

And here, we have the effect of heat on mouse protective activity of saline extract incubated at the temperatures indicated. This is unheated, 65 degrees, 70, 75, 80, and at three different concentrations of saline extract.

You can readily see that at 20 microgram dose level, there is some marked destruction of the protective activity. This cannot be shown as well except at the 80 degree level.

May I have the next slide? I think it will show more dramatically what happened at the one-dose level. (Slide 12)

This is at the 20 gamma dose of saline extract. As you can see, the activity drops rapidly with temperature and even at 60 degrees, you can hardly notice a destruction of the protective activity.

The protective antigen must have a considerable amount of protein since activity also correlates a high protein containing peak in our hands. It might also contain fatty acids, and if I were to make a guess at present, I would say that the protective antigen is most likely a/~~lipid protein~~ lipoprotein of some kind, but this still remains to be determined.

The protective antigen as extracted by the method that we have described is ~~partly dispersed~~ polydispersed. It sediments in the ultracentrifuge, but it takes long periods of centrifugation to sediment the activity.

The results of these studies are illustrated in the next slide which shows the effect of ultracentrifugation on mouse protective activity of saline extract. (Slide 13)

This (indicating) is the time in hours, three hours, six hours, sixteen hours. This is the protective activity of the sediment. This is the protective activity of the supernatant.

As you can see, at three hours, the protective activity is distributed both in the sediment and in the supernatant.

These are speeds, of course, that sediment molecules of protein. At six~~teen~~ hours, most of the activity is found in the sediment, but still considerable activity can be found in the supernatant.

And finally, at sixteen hours, most of the activity is found in the sediment. The protective antigen is negatively charged at pH 7, and our purest preparations obtained by starch electrophoresis always possess histamine sensitizing activity.

In fact, the results that I have described to you could also be applicable to histamine sensitizing factor.

Summarizing our present results on the nature of the protective antigen, we can say the following:

Next slide, please. (Slide 14)

The protective antigen is found in the cell walls. It can be solubilized by various methods. It is stable when in the cells or in lyophilized form. It is polydispersed, and it has a net negative charge at pH 7. It is relatively heat labile.

I must emphasize "labile" since I made a mistake yesterday of saying the opposite. It is destroyed by heat.

It is also destroyed by formalin. It is probably a protein containing lipid, although this is still a questionable observation.

It sensitizes mice to histamine and to anaphylaxis.

And finally, in our hands, our purest preparations still show a little toxicity, but this point will have to be clarified in the future.

Thank you.

(Applause.)

DR. ELDERING: Thank you, Dr. Munoz. It is very encouraging work.

I shouldn't have been so pessimistic in my opening remarks.

The next paper, "Protective ~~antigen~~ Antigen/of B. pertussis Released by Sonic Energy," will be given by Dr. A. C. Wardlaw.

Dr. Wardlaw.

DR. WARDLAW: Dr. Eldering, ladies and gentlemen:

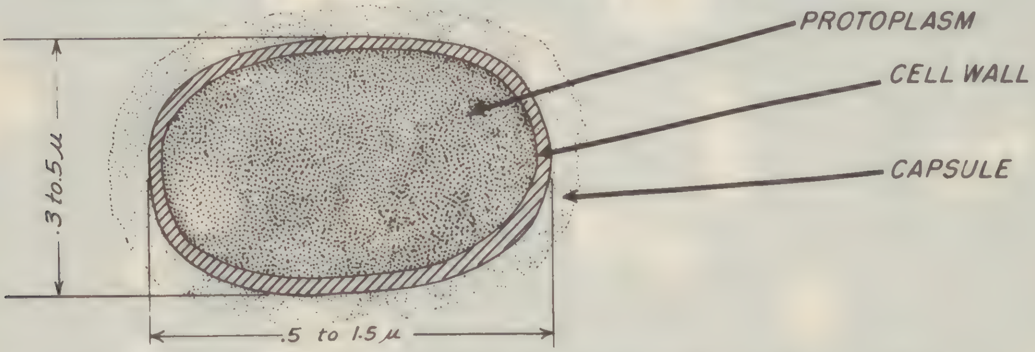
(Slide 1)

Slide 2

(Slide 3)

(Slide 4)

DIAGRAM OF TYPICAL BORDETELLA PERTUSSIS CELL



TENTATIVE LOCATIONS OF ANTIGENIC COMPONENTS

CAPSULE:

AGGLUTINOGEN (?)

HEMAGGLUTININ (?)

CELL WALL:

PROTECTIVE ANTIGEN

HISTAMINE SENSITIZING FACTOR

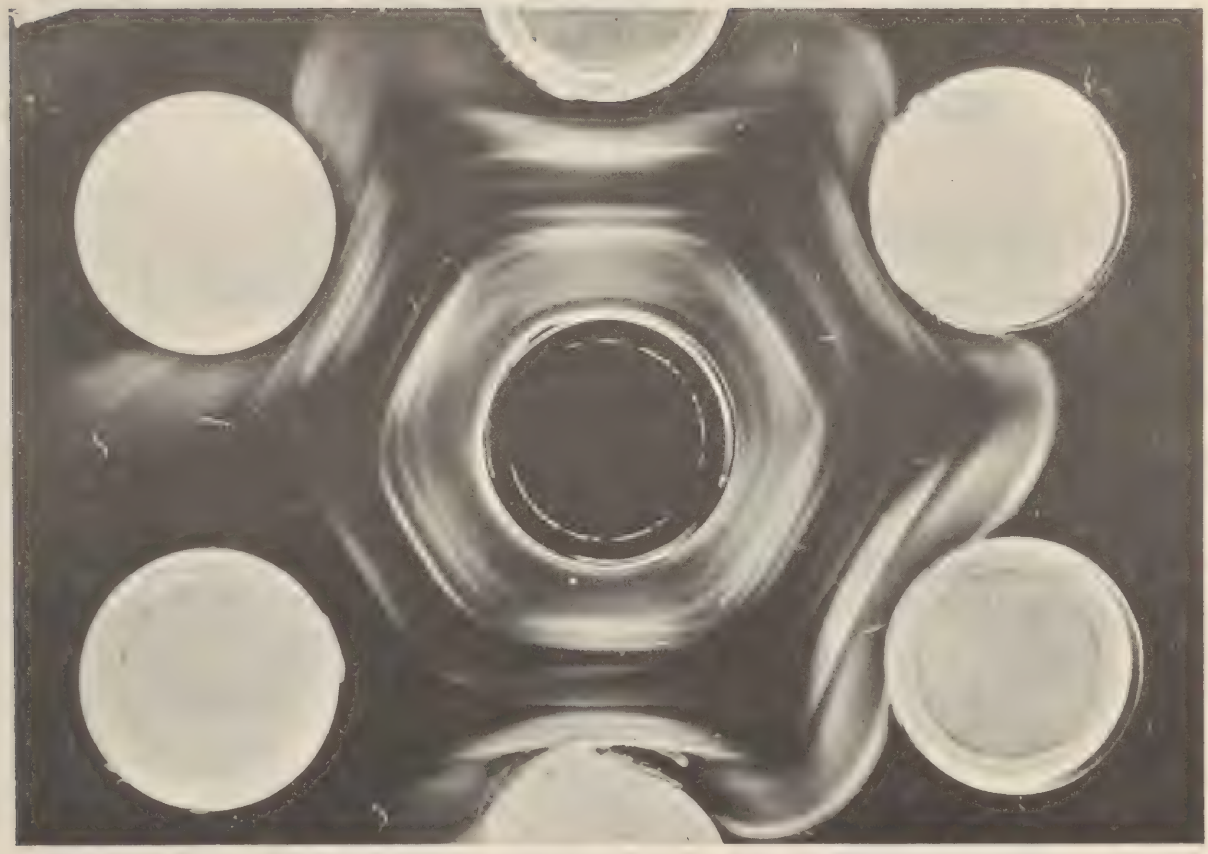
ENDOTOXIN

PROTOPLASM:

HEAT LABILE TOXIN

MANY OTHER ANTIGENIC COMPONENTS

(Slide 5)



(Slide 6)

Method of Preparing Acetone Dried Cells

Whole live cells (1000 B/ml) + 3 volumes acetone and filtered

cell paste | acetone discarded

+

3 volumes acetone
(same volume as before)

cell paste | acetone discarded
dried and finely ground

(Slide 7)

Method of Preparing Saline Extract from Acetone Dried Cells

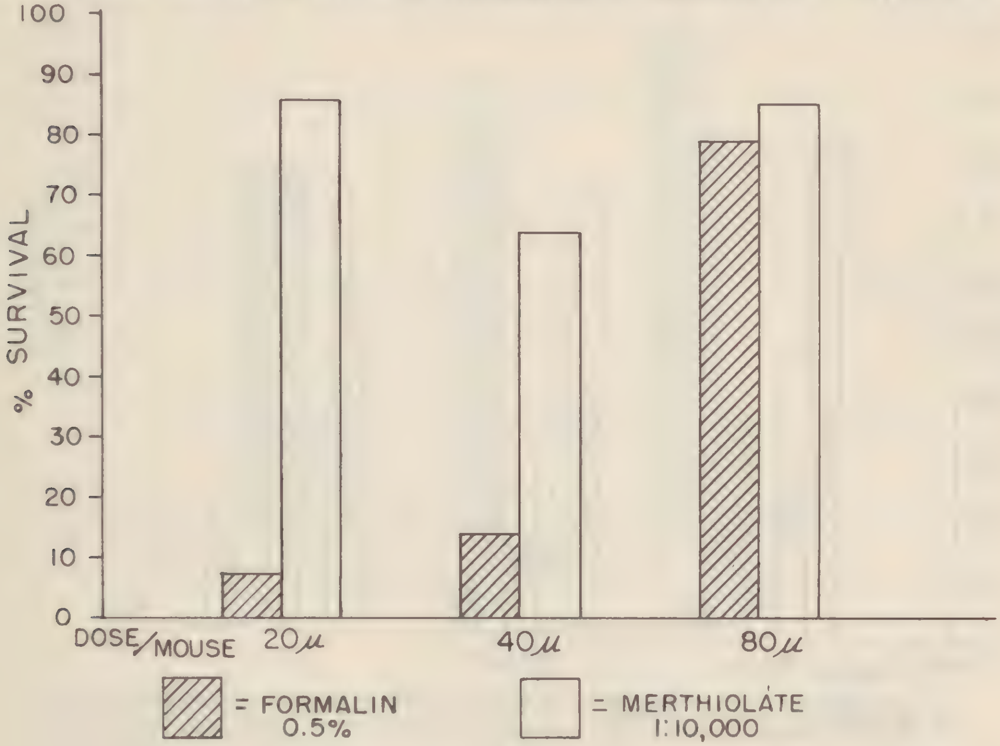
1) 15 g dried cells suspended in 500 ml of saline.
2) Homogenized and pressurized. Add 500 ml more of saline.
3) pH adjusted to 8.5. Incubated overnight at 2-5^{o}C.
4) Centrifuged at 27,000 g for 40 min.
5) Supernatant is saline extract (SE).
6) The sediment can be re-extracted two times more with 200-400 ml of saline, recentrifuged and the supernatants added to SE in step 5.

(Slide 8)

Chemical Analysis of Saline Extract from *B. pertussis*

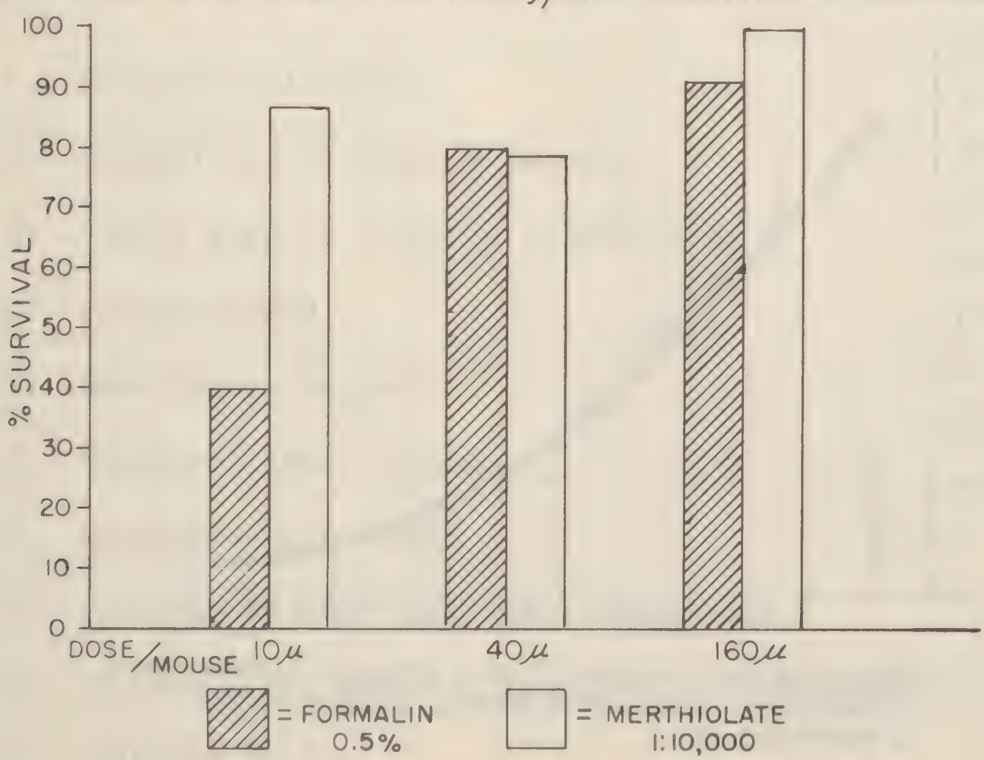
Compound	Percent in Sample
Nitrogen	14.6
Hexose	4.5
Hexosamine	0.4
Phosphorus	1.8
Fatty Acid Esters + Fatty Acid Amides	28.4

EFFECT OF FORMALIN AND MERTHIOLATE ON MOUSE PROTECTIVE ACTIVITY OF SALINE EXTRACT (0.8 mg/ml INCUBATED AT 37° FOR 1 WEEK)



(Slide 9)

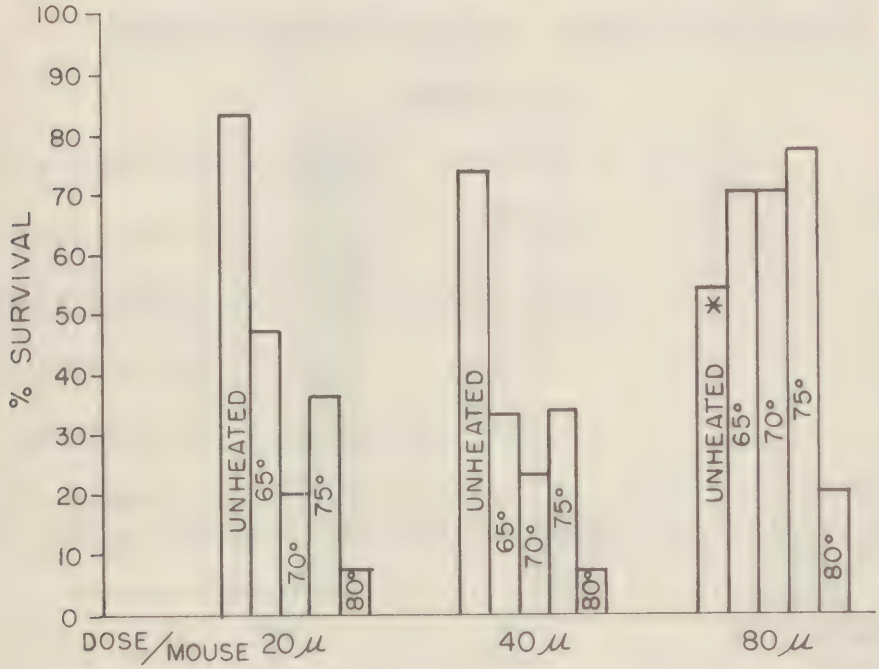
EFFECT OF FORMALIN AND MERTHIOLATE ON MOUSE PROTECTIVE ACTIVITY OF SALINE EXTRACT (1.6 mg/ml INCUBATED AT 37° FOR 1 WEEK)



(Slide 10)

(Slide 11)

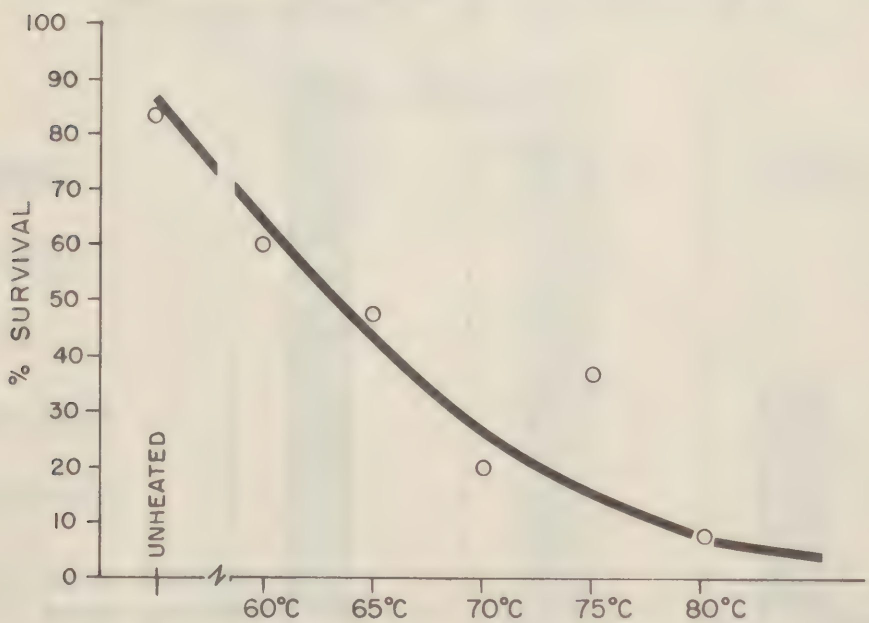
EFFECT OF HEAT ON MOUSE PROTECTIVE ACTIVITY OF SALINE EXTRACT
(HEATED TO TEMPERATURE INDICATED FOR 1/2 HOUR)



* SLIGHTLY TOXIC DOSE

EFFECT OF HEAT ON MOUSE PROTECTIVE ACTIVITY OF SALINE EXTRACT
(HEATED TO TEMPERATURE INDICATED FOR 1/2 HOUR)

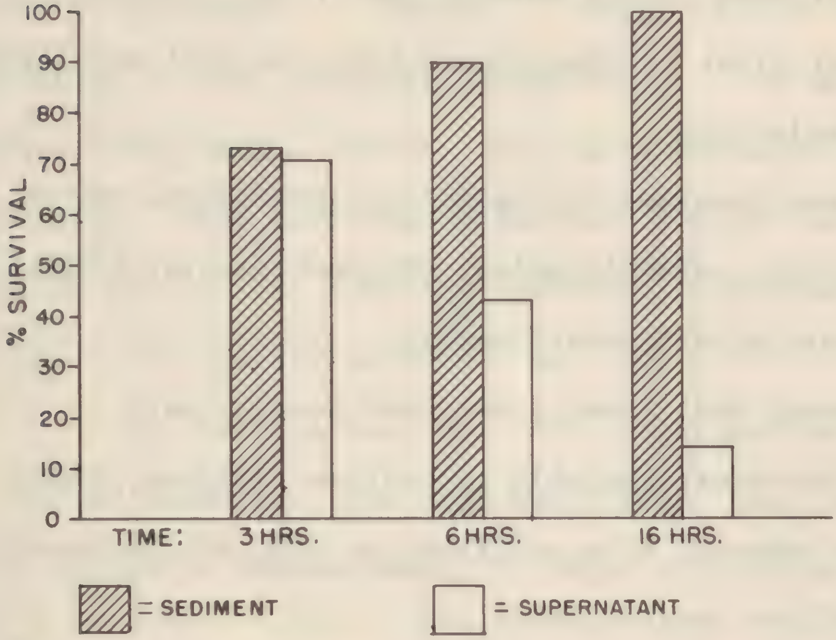
(Slide 12)



*EACH OF 15 MICE RECEIVED I.P., 20μ OF SALINE EXTRACT 14 DAYS
I.P. CHALLENGE WITH APPROXIMATELY 40,000 VIRULENT
B. PERTUSSIS CELLS.

(Slide 13)

EFFECT OF ULTRACENTRIFUGATION ON MOUSE PROTECTIVE ACTIVITY OF SALINE EXTRACT



A SOLUTION SALINE EXTRACT CONTAINING 2 mg/ml WAS CENTRIFUGED AT 125,000g FOR LENGTH OF TIME INDICATED. THE SAMPLES WERE TESTED AT AN EQUIVALENT CONCENTRATION OF 80μ PER MOUSE (1/5 DILUTION)

(Slide 14)

Properties of Protective Antigen

1. Found in cell walls
2. Solubilized by various methods
3. Stable when in cells or lyophilized
4. Polydispersed
5. Negatively charged
6. Relatively heat labile
7. Destroyed by formalin
8. Probably is a protein containing lipid
9. It sensitizes mice to histamine and anaphylaxis
10. Slightly toxic

I think most students of pertussis would agree that before any effective attempt can be made to purify the protective antigen, it must first be dissociated from the cell and liberated in a truly soluble form.

Various chemical and mechanical procedures may be used to effect this solubilization, the most popular being exposure to sonic or ultrasonic energy.

Although sonic energy has been used by many investigators who wanted soluble protective antigens, there seem to be few adequate data published on some of the quantitative aspects of the sonic process.

The starting point of the present investigation was, therefore, to explore such questions as: Can the pertussis cell be dissolved completely by continuing the sonic treatment for long enough? If not, is it at least possible to dissolve all of the protective antigen? When is the protective antigen released from the cell in relation to the dissolution of other factors such as toxin and dry weight?

In other words, is it possible by using either a long exposure or a short exposure to get preferential solubilization of the protective antigen and partial separation from other cellular components?

And finally, is it advantageous to do the sonic treatment in the presence of such an additive as versene or desoxycholate which may promote cell lysis.

So far, my studies have all been made with a single strain of pertussis -- (18334) -- which is used for vaccine production in the Connaught Laboratories. It was grown in casamino acid medium and the live cells collected on a Sharples centrifuge. They were then dispersed in 2 per cent casamino acids at pH 8.0 with a Waring blender to a concentration between 500 and 1,000 opacity units per ml.

The machine used for sonic treatment was the Raytheon DF-101, 10 Kilocycle, 250 watt oscillator, the treatment vessel being cooled to ~~130 degrees~~ 1° C with water circulated from a cold bath.

In carrying out the sonic treatment, I attempted to meet several requirements. Maximum number throughput of cells, maximum disruption and adequate high speed centrifugation to remove the cell fragments from the sonic lysates.

The scheme used is shown in the first slide.

I started with 100 ml of 900 opacity unit of live cells and gave them 30 minutes sonic treatment for one hour at 20,000 g.

The g at the bottom of the tube would be about 27,000. This is in the middle, and this gave a supernatant residue.

This residue which I now regarded as rather more resistant than the starting material was given 60 minutes sonic treatment followed by centrifugation to give a second supernatant and a second residue. And this residue was given 120

minutes sonic treatment to give a third supernatant and a final residue. So this final residue had 210 minutes of sonic treatment altogether.

The intention behind this scheme was to avoid excessive sonification of the readily dissolved components which were separated off by centrifugation at intervals while giving prolonged treatment to the more refractory parts of the suspension.

Now, each of these fractions was dialyzed against water to get rid of the casamino acids and were resuspended to 100 ml. So that we have the same equivalent of opacity unitage in each of the fractions as we have in the starting material.

(Slide 2)

The next slide shows the changes ~~inopacity~~ in opacity which took place during sonication. This is a plot on log paper putting the per cent of the original opacity against the overall elapsed time of sonic treatment. And you can see that after 30 minutes, the opacity was down to one-half. After 90 minutes, it was down to about 25 per cent of the original. And it kept on going down.

These two points represent two independent experiments, one of which was run for 210 minutes according to the scheme I have just shown, and the other was continued for 400 minutes.

I think the important thing to note is that it seems that 90 minutes of sonification gives 75 per cent of the material dissolved. In other words, 25 per cent in the residue. And it

looks as if one were to continue sonic treatment for an extravagantly long time that perhaps eventually all the cell would dissolve. But to get the last few per cent dissolved would take excessive time.

(Slide 3)
The next slide shows some chemical data on the soluble fractions and on the final residue. And all these figures are expressed in terms of the starting quantity of live cells.

Now, the sonic supernatant after the first 30 minutes contained about one-half of the starting dry weight. The effect of another 60 minutes of sonic treatment was to dissolve a further one-quarter. And the effect of another 120 minutes of sonic treatment was to dissolve a further one-eighth.

So clearly, as the sonic treatment continued, one got diminishing returns in terms of solubilization. In other words, by ~~pulling~~ pooling the three soluble fractions, approximately 80 per cent of the cell was made soluble, leaving a residue of about 20 per cent of the starting dry weight.

If you look at the analyses for protein and DNA, you see a rather similar picture -- about one-half dissolved after 30 minutes, then further quarter in the next hour, and a further eighth or tenth in the further two hours of sonic treatment, giving altogether around about 70 or 95 per cent in the supernatant and these amounts in the final residue.

The amount in the pooled supernatants and final

residues should add up to 100 per cent, the differences shown here being due to experimental error or losses in dialysis and so on.

The same fractions on which we have this chemical information were next examined for toxicity.

Next slide, please. (Slide 4)

This is just a very abbreviated sort of test -- just groups of three mice injected with these numbers of equivalent opacity units of the various fractions, and the results expressed as survivors over total.

I think the interesting thing is that the sonic supernatants obtained in the first two centrifugations were more toxic than the whole cells that I started out with. This is, of course, a well-known observation.

There was very little toxin released during the final two-hour period of sonic treatment, and the final residue was nontoxic at the 10 opacity unit level.

All the fractions collected in this experiment were tested for protective activity, using the intracerebral mouse protection test and comparing the potency of each fraction with the NIH standard vaccine.

The results were evaluated by protein analysis and are expressed on the next slide as the relative potency with respect to the NIH standard vaccine and in brackets the 95 per cent confidence limits. (Slide 5)

This relative potency, of course, is on the basis of equal equivalent opacity units or fraction and NIH vaccine. Obviously, with confidence limits as wide as these, one cannot push any quantity of interpretation of the data too far. Nevertheless, certain conclusions can tentatively be reached.

It looks as if the starting cells were significantly slightly more potent than the NIH vaccine. This figure of 2.8 means 2.8 times more potent. But the lower confidence limit is only just about 1.0.

After 30 minutes sonic treatment, the activity appears to be distributed rather evenly between the supernatant and the residue, perhaps more in the residue. Sixty minutes sonic treatment liberated a further useful quantity of antigen in the supernatant, but still left a residue that had some activity. And 120 minutes, this is a very low figure for protective antigen. Here, it is about .23. So there is not much protective antigen being released during this rather long period here.

And I think the interesting thing is that the final residue after 210 minutes treatment still has some protective antigen in it.

I think that these figures correspond rather closely with the figures for release of dry weight. In other words, approximately half of the original activity released in the first step, perhaps a further one-quarter and a further one-eighth, leaving a certain amount behind in the final residue.

This shows, I think, that the protective antigen is rather stable to long periods of sonic treatment if you consider that this material has had 210 minutes, and we have found that even after 400 minutes of sonic treatment, the final residue still has some activity.

Nevertheless, if one pools these supernatants, one does get a soluble preparation which has a potency similar to NIH standard vaccine. And I think it isn't worthwhile to try to extract the last little bit of antigen because of the inordinately long time of treatment that is required.

We have made the tacit assumption so far that it is more efficient to interrupt the sonic treatment at intervals and remove the soluble fraction by centrifugation rather than simply centrifuge once at the very end of treatment.

To test this directly, an experiment was made to compare 210 minutes of continuous sonic treatment with 210 minutes interrupted at 30 and 90 minutes as in the experiment just described. The results are summarized in the next slide which suggests that the interrupted procedure was considerably more efficient. (Slide 6)

This is the amount of dry weight protein and so on in the supernatant after 210 minutes of continuous sonic treatment followed by one centrifugation (indicating), and this is the pooled supernatant from the stepwise procedure.

There is much more dry weight liberated, much more

protein, much more toxicity. In fact, it looks as if the sonic treatment for 210 minutes may have destroyed some of the toxin here, and the protective antigen relative to NIH vaccine showed a relative potency of 1.0 here. And these are the individual supernatants.

They actually add up to 1.5, which would be significant relative to this.

So we don't know for sure that there is more antigen released by this interrupted process, but this may be because of insensitivity of the antigenicity test.

I think there is a good reason for the greater efficiency of the interrupted process based on viscosity considerations. The sonic disruption of bacteria depends on the sheer forces being set up between the bacteria and the surrounding medium. There has to be relative motion of the bacteria with respect to the medium.

This motion in turn is impeded and made less violent if the medium is viscous. Thus, one would expect there to be some advantage in keeping the viscosity low by periodically removing the DNA and other viscous components of bacterial lysis.

We now come to the question of whether it is advantageous to do the sonic treatment in the presence of additives such as desoxycholate or EDTA which may promote lysis, or merthiolate which may help to stabilize the antigen.

An experiment was, therefore, made in which a batch of cells was divided into portions which were sonically disrupted under identical conditions except that different additives were put in just before the start of sonic treatment. Some chemical data are shown on the next slide. (Slide 7)

This experiment was done using less cells, half the quantity of cells, and a lower opacity than in the previous experiment, and there was just one cycle of treatment, just one hour, and it looks as if there is an overall much more efficient dissolution which I think is due to this lower quantity of material being treated.

But the thing to note is that there is very little difference between the various things that were measured in the ~~new~~ no additive control and in the presence of these various additives. That is to say, the reduction in optical density was the same and liberation of dry weight into the supernatant, liberation of protein into the supernatant, liberation of DNA into the supernatant.

There is just one point that the desoxycholate process seemed to do a better cleaning out of DNA in the residue because this value here (indicating) does seem to be significantly lower than the DNA in these other residues.

The same sonic supernatants were examined for various biological activities in the last slide. (Slide 8) And, again, there is some difference between the desoxycholate material and the

others.

If you look at the toxicity figures, survivors over total, at these number of opacity units per mouse, the desoxycholate sample is nontoxic at the 10 opacity unit level.

This is a misprint here. It should be zero out of three which makes the versene/sample essentially the same as the merthiolate and no additive control.

With regard to the desensitizing factor, mice injected with these levels, and again survivors over total, there doesn't seem to be any significant difference between the various fractions. They are all showing good histamine sensitization at the two opacity unit level. And apart from this one here (indicating) which seems to have higher activity, they are showing no histamine sensitization at this lowest level tested.

In the protective antigen relative to NIH standard, there isn't much difference between the different samples. It is rather interesting that the protective antigen can withstand an hour of sonic treatment in the presence of these surface active agents if the antigen is a lipoprotein.

I would like to finish with some consideration of a point I passed over rather lightly, and that is the question of what we understand by the word "soluble."

For purposes of this study, I have taken "soluble" to mean not sedimented during one hour at 20,000 g. More

recently, however, I have begun to question the adequacy of this as a result of ultracentrifuge studies.

When we took the 20,000 g supernatants and centrifuged them for five hours at 70,000 g, we got a gelatinous precipitate and an absolutely clear yellow supernatant which looked like nutrient broth whereas the starting material, the 20,000 g supernatant, looked rather like a clear serum.

By the mouse protection test, we found that about two-thirds of the protective antigen was in this water-clear supernatant and one-third in the pellet. What we are hoping now is that the 70,000 g supernatant contains the protective antigen in a truly soluble form which may be satisfactory for chemical fractionation. And studies are under way to find out something about the molecular weight of the antigen in the 70,000 g supernatants.

Thank you.

(Applause.)

DR. ELDERING: Thank you, Dr. Wardlaw.

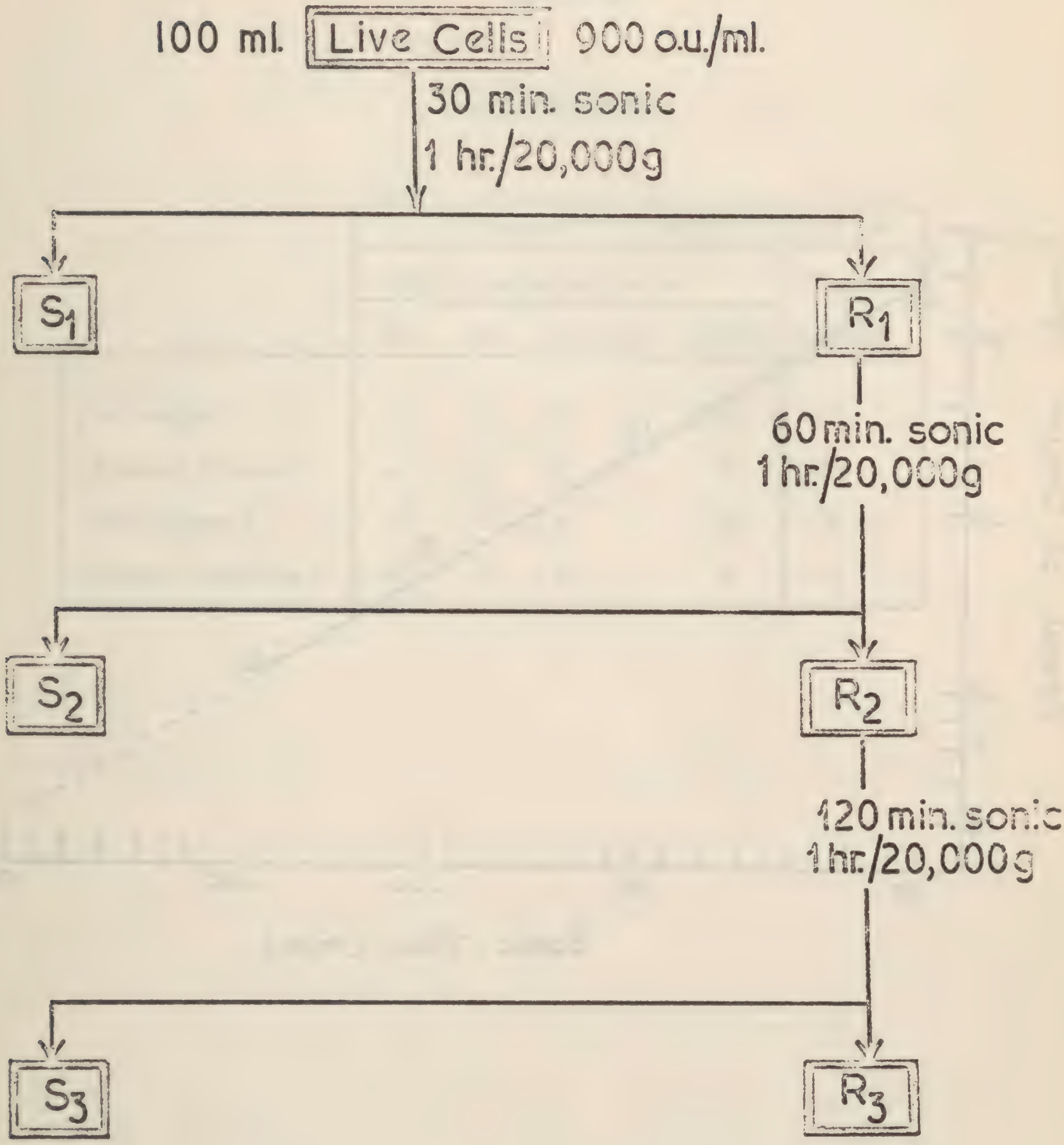
We will pass on now to the next paper, "Some Unsuccessful Attempts to Produce a Soluble Pertussis Vaccine", by Mr. Leo Levine.

Mr. Levine.

MR. LEVINE: Dr. Eldering, ladies and gentlemen: Our initial approach to the development of a soluble pertussis vaccine has been to avoid deliberate cell disintegration and to

A.C. Wardlaw (1)

(Slide 1)



A.C. Wardlaw (2)

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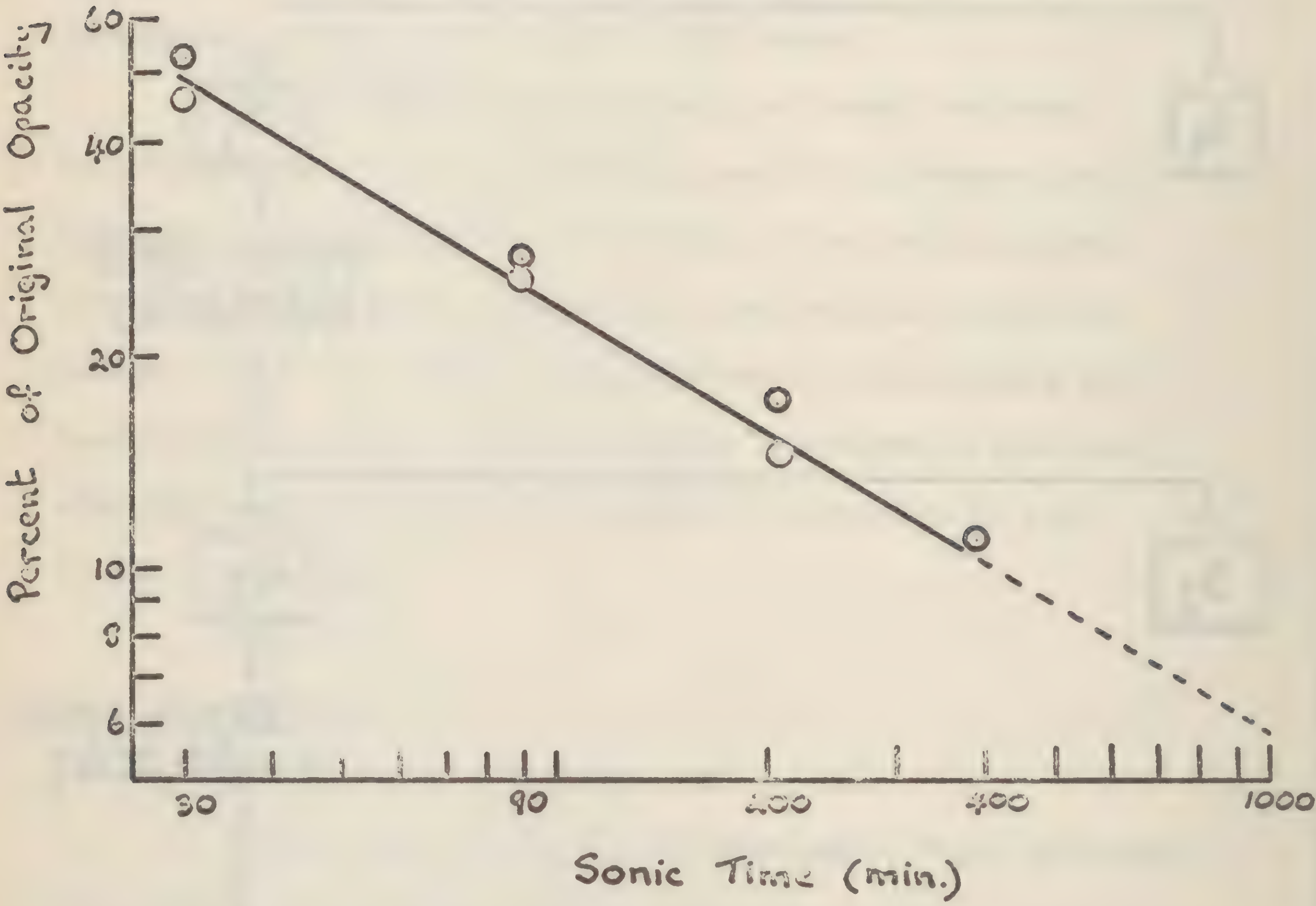


A.C. Wardlaw (3)

(Slide 3)

Chemical Data

	Percent of starting material in				
	Sonic supernatant at :-				Final
	30	90	210 min	Total	Residue
Dry weight	48	24	12	84	22
Protein (biuret)	40	22	8	70	16
DNA (Dische)	57	29	9	95	20
Hexose (Anthrone)	27	3	2	32	56

A. C. Wardlaw (4)

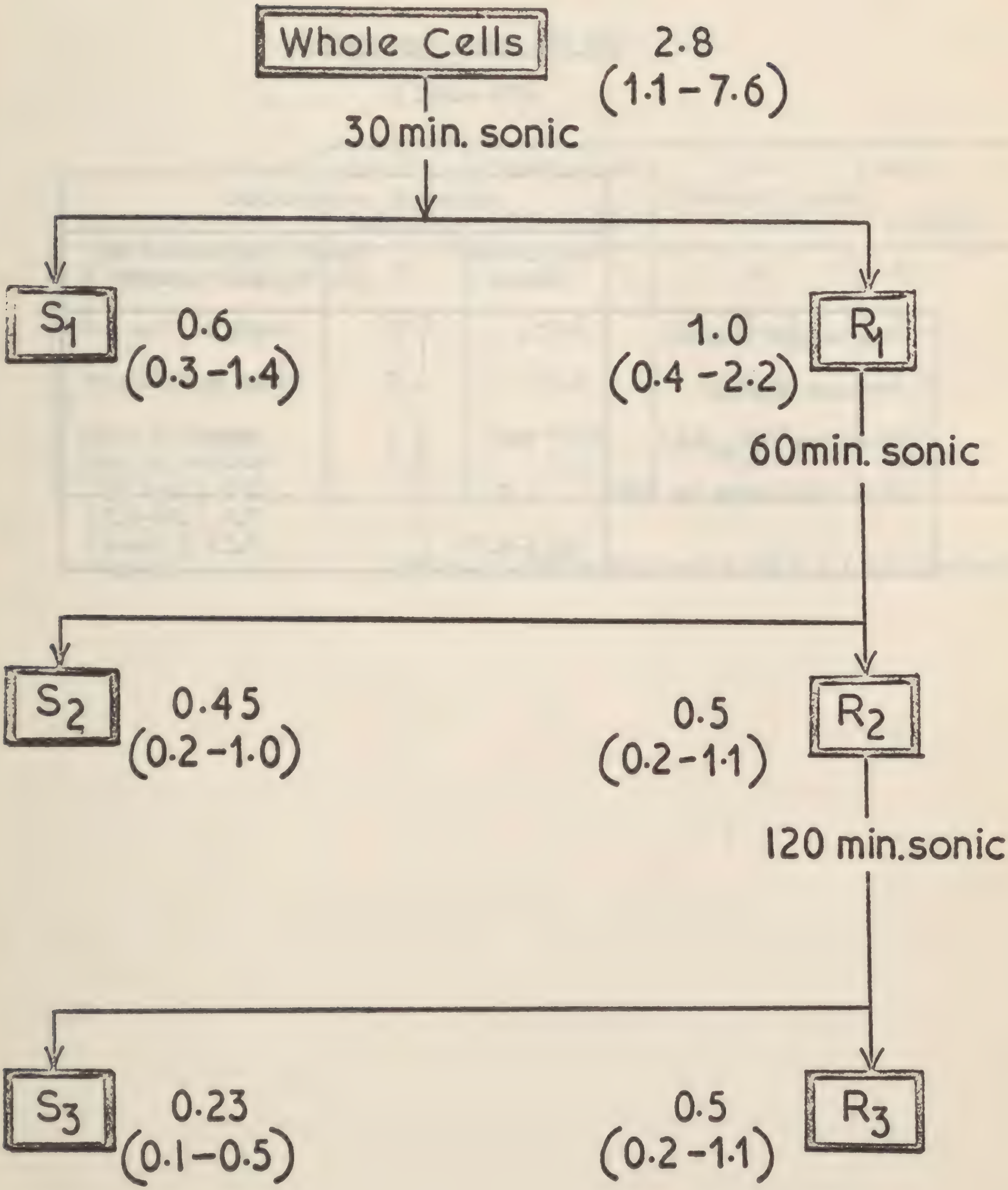
(Slide 4)

Toxicity Tests

Dose per mouse (o.u.)	Survivors/total injected with				
	Whole cells	Sonic supernatant at 30	90	210 min	Final residue
0.4	3/3	0/3	3/3	3/3	3/3
2.0	3/3	0/3	0/3	3/3	3/3
10.0	0/3	0/3	0/3	2/3	3/3

A.C. Wardlaw (5)

(Slide 5)



A. C. Wardlaw (6)

(Slide 6)

210 min Sonic Treatment

(870 ou/ml)

	Amount in supernatant	
	Continuous sonic	Sonic interrupted at 30, 90, 210 minutes
Dry weight (mg/ml)	11	17.5
Protein (mg/ml)	7	13.5
Toxicity (LD_{50}/ml)	700	2400
P.A. (relative to NIH)	1.0 (0.3-2.9)	1.0 (.4-2.9) 0.4 (.20-1.2) 0.13 (.04-.4)

A.C. Wardlaw (7)

(Slide 7)

Additives

	No Additive	Merthiolate (0.1 mg/ml)	Na Desoxycholate (5 mg/ml)	EDTA (0.0025M)
% Redn. in O.D.	83	83	86	83
Dry wt* in super	10.1	9.6	11.3	10.6
Protein* in super	7.1	6.9	6.4	7.8
DNA* in super	1.1	0.83	1.2	1.1
DNA in residue	0.4	0.3	0.06	0.4

* mg/ml (50 ml of 570 ou/ml in CAA pH 8.0 1 hr treatment)

A. L. Wardlaw (8)

(Slide 8)

Additives (cont)

		No Additive	Merthiolate (0.1 mg/ml)	Na Desoxycholate (5 mg/ml)	EDTA (0.0025M)
Toxicity S/T at o.u. /mouse	0.4	3/3	3/3	3/3	3/3
	2	1/3	2/3	3/3	3/3
	10	0/3	0/3	3/3	0~~3~~/3
H.S.F. S/T at o.u. /mouse	0.08	4/4	4/4	1/4	4/4
	0.4	2/4	3/4	2/4	2/4
	2	1/4	1/4	0/4	0/4
P.A. (relative to NIH Std)		1.8 (0.8-4.2)	1.1 (0.5-2.5)	1.6 (0.8-3.4)	2.9 (1.3-6.6)

concentrate on factors responsible for the presence of protective activity in vaccine supernatants and in saline washings which have been widely reported. We felt that cell disintegration may liberate cytoplasmic toxins and complicate subsequent fractionation.

Since the chemical nature of the protective antigen is not yet known, we decided to treat cells with enzymes that attack proteins, polysaccharides, and lipids on the assumption that the protective antigen would be destroyed by enzymes to which it was a substrate, and perhaps be liberated by enzymes to which it was not a substrate. This would give us a clue to its structure and at the same time a possible indication of a practical method.

The costly and time-consuming standard mouse assay for potency was incompatible with the fairly large-scale screening of several treatments applied to several vaccine strains and so we adopted an essentially qualitative test in which we used 10 mice for each material at a single dose of 1.7 Opacity Equivalents (OE), otherwise following the intracerebral challenge method. This test was interpreted as negative with up to three survivors, promising with seven or more, and equivocal with four to six.

The experimental vaccines were grown on Cohen and Wheeler medium for 72 hours in eight liter volumes with vigorous aeration. Cultures were killed with 1:10,000

Merthiolate. Cells were collected by continuous centrifugation on the refrigerated Servall at 37,000 X G. They were resuspended in water, shell frozen and lyophilized for storage.

To carry out a test, cells were weighed out, resuspended in appropriate buffer with the aid of the Waring blender, and the test reagents added. Enzyme treatments were carried out for 18 hours at 37 degrees Centigrade.

Eight treatments were enzymatic, the enzymes being papain, lysozyme, trypsin, pancreatin, amylase, lipase, cellulase, and diastase. The reagent mixture thiourea, urea, and formamide (TUF) constituted a ninth treatment. These treatments were all applied to the reconstituted lyophilized cells for 10 lots of vaccine involving 6 different strains at nearly optimum conditions for each reagent. The screening test in mice was applied to the soluble phase, separated at 27,000 g, and to the resuspended residue. As controls, the supernatant and residue of each vaccine were similarly tested. Including this control, 10 treatments were thus applied to 10 lots, giving a 10 by 10 table for extracts and residues, of which the marginal sums are believed to have quantitative value.

Could I have the first slide, please?

In this column, we have the percent survival for the summed tests for 10 lots, except an occasional lot is missing due to its material.

Percent survival for the extracts and for the

residues, the overall percent survival for extracts, was 34 per cent and for residues 64 per cent.

We have in these columns the rank order of the various treatments, the first rank being diastase with 48 per cent survival for that enzyme. It is very significant that the second ranking material sharing its rank with trypsin is the original vaccine supernatant showing an overall survival for 100 mice of 40 per cent, and the residue being 72 per cent.

As to the differences here, the only pair that have a significant difference at the 5 per cent level is the worst extract, TUF, at 21 per cent, and the best, 48 per cent. All the other differences are not significant.

It is of interest that in no case did any treatment completely destroy protective activity.

The ~~treatest~~ greatest destruction occurred with the one treatment that was not enzymatic, indicating the antigen is better protected against enzymes than against the protein denaturant, urea.

The original supernatants had the same order of potency as the treatment extracts, and in fact ranked second, along with trypsin, which were exceeded only by diastase.

The persistent evidence of activity in vaccine supernatants led us to make further quantitative comparisons between supernatants and whole cultures. If a reasonable amount of protective activity could be demonstrated in the supernatants,

it would be worthwhile to attempt concentration, isolation, and characterization from this solution, even though the amount might not be adequate for routine public health application. A number of different lots were used for the comparison in mouse assays performed over a period of six months.

A total of 855 mice were used for the comparison, with the finding that supernatants ranged in potency from about 10 to 30 per cent of whole culture vaccines. There was some indication that supernatants of older vaccines were more potent than those made more recently, although vaccines of only three months of age had detectable protective activity in the supernatants.

A large number of efforts were made to concentrate the protective activity in the supernatants by ammonium sulfate (AS) precipitation. The soluble starch that is added to liquid medium is irreversibly precipitated by AS. Elution of these precipitates was carried out with sodium phosphate.

At this point, we decided to omit the addition of soluble starch to the medium of our experimental vaccines and found no change in potency, confirming the work of Kuwajima, et al.

These starch-free supernatants gave very much smaller recipitates at 50 or 90 per cent saturation with AS, which only partially redissolved. These were also eluted by dialysis against phosphate.

These eluates represented concentrations of the original supernatants of the order of 50-fold or more, yet the mouse protective activity was not recovered beyond a few percent. About a dozen experiments of this type were performed, yielding disappointing results. In some cases, 0.5 per cent human albumin was added to the supernatants to afford a reversibly precipitable carrier, but the results were the same.

Last January the work of Barta on a soluble pertussis antigen using desoxycholate became available to us. We immediately undertook to confirm this work. By February 21, we had placed two extracts on test, one from a 48-hour culture, the other 72-hour. His procedure was closely followed.

On March 14 two further extracts were ready and placed on test. As controls, we used the supernatants of merthiolate killed portions, the original cultures at dosages equivalent to those of the test extracts. The results with three of the four extracts -- may I have the next slide, please?-- showed significantly greater potency for the original supernatants than for the extracts. (Slide 2)

On May 9 the one favorable extract was retested and this time found to be inferior to original supernatant. All these tests were complete bioassays, using three doses with 10 or 16 mice per dose.

Our criterion of a successful treatment is that its soluble extract must be significantly more potent than the

supernatant of its parent culture. Otherwise there is no evidence that the treatment achieved anything. On this basis, we were unable to confirm this method. Desoxycholate was previously found ineffective in other methods by Millman, Schuchardt and Gray.

A variety of additional experiments were also carried out during the past year. Extractions were attempted with phenol at high concentrations according to Westphal and at low concentrations according to Behrens and Ensminger with ethanol and ether. Concentration from supernatants was attempted using TCA, zinc salts, $Al\ PO_4$ precipitation, and by simple pervaporation and dialysis. The results were in no case encouraging.

Our efforts in the future will be the application of column chromatographic techniques for the fractionation of starch-free culture supernatants. Some preliminary runs have indicated interesting differences in the effluent spectra of vaccine supernatant and parent medium run on a DEAE column.

Eldering has pointed out that ever since bacterial vaccines were first used, workers have been searching for ways to separate the essential immunizing antigen from the rest of the bacterial cell. Yet in spite of decades of competent work in this direction, little application has been made in the actual preparation of immunizing agents. Recent work on this phase of pertussis vaccine methodology is characterized by frequent claims and a paucity of confirmation.

Our work in part has recapitulated the experience of Millman, and others who attempted in vain to confirm a long series of optimistic reports involving treatments based on such reagents as desoxycholate, lauryl sulfate, lysozyme, ether, TUF, phenolics, as well as various methods of mechanical cell disintegration.

Most workers compare their soluble extracts with the whole parent vaccine and are often satisfied with a certain relative potency. I should like to emphasize that the comparison should also be made with the vaccine supernatant, and unless the test extract can be shown to be significantly better than the parent supernatant, there is no evidence the treatment accomplished anything.

Thank you.

(Applause.)

DR. ELDERING: At this point, I am tempted to quote poetry and say, "Fear not, the struggle now availeth."

Dr. Murray says we may have a coffee break at this point.

(Whereupon, a recess was taken.)

DR. ELDERING: We will now resume, and we will hear from Dr. Guerault on the interesting topic, "Protective and Histamine-sensitizing Activities of Pertussis Vaccine and Various Fractions."

Dr. Guerault.

Table 1. Summed mouse survival rates in potency estimates on extracts and residues of 10 vaccine lots (6 strains) each submitted to various treatments.

Treatment	Extract			Residue			Extract + Residue		
	No. of mice	% Survived	Rank order	No. of mice	% Survived	Rank Order	No. of mice	% Survived	Rank Order
Original vaccine	100	40	2	39	72	4	139	49	5
T U F	100	21	8	73	44	10	173	31	10
Papain	99	28	6	78	65	7	177	45	8
Lysozyme	100	33	4	79	62	8	179	46	7
Trypsin	99	40	2	80	69	5	179	53	3
Pancreatin	100	39	3	80	48	9	180	43	9
Amylase	90	39	3	68	74	3	158	54	2
Lipase	80	26	7	60	78	1	140	48	6
Cellulase	80	30	5	59	76	2	139	50	4
Diastase	90	48	1	70	66	6	160	56	1
Total	938	34		686	64		1624	47	

Controls (no treatment) 90 mice, 18% survived 1/50 challenge dose.

Controls (whole vaccine) 87 mice, 86% survived full challenge.

T U F: Thiourea, urea, and formamide.

TABLE 2. MOUSE POTENCY TESTS ON DESOXYCHOLATE EXTRACTS OF B. PERTUSSIS COMPARED WITH MERTHIOLATE KILLED PARENT WHOLE CULTURE AND CULTURE SUPERNATANT.

DOSES: .08, .4 AND 2.0 OPACITY EQUIVALENTS

STRAIN	WHOLE CULTURE			SUPERNATANT			0.5% D O C		
22490; 48 HR.	1/10	7/10	10/10	0/10	1/10	6/10	1/10	0/10	2/10
22490; 72 HR.	2/9	5/10	10/10	1/9	0/10	7/10	0/10	1/10	1/10
18,628; 48 HR.	6/10	5/7	9/9	2/10	2/9	4/10	5/9	5/10	6/8
18,628; 72 HR.	2/9	2/9	10/10	3/10	4/10	8/10	1/10	2/10	9/10
18,628; 48 HR.				4/16	4/16	10/16	2/16	0/16	2/14
TOTAL	11/38	19/36	39/39	10/55	11/55	35/56	9/55	8/56	20/52
% SURVIVORS	29	53	100	18	20	63	16	14	38

GUÉRAULT

DR. ~~GEURAULT~~: Dr. Eldering, Dr. Pittman, ladies and gentlemen: Little is known of the true nature of the histamine-sensitizing factor and the relations of HSF to other pertussis antigens. A correlation has been referred to in the past between the histamine-sensitizing properties of pertussis vaccine in mice and their protective ability in children.

Slide one is with minor modifications a table presented last year at the Prague conference on pertussis.

The figures from left to right correspond to the order of decreasing activity as calculated for ten out of the vaccines used in the British Medical Research Council field trials and tested in the laboratory by the four methods indicated -- that is, agglutinins in children, agglutinins in mice, mouse protection test, and histamine-sensitization test.

The data for the last-mentioned test are from Maitland, et al., 1955. These vaccines did sensitize mice to histamines with various intensities, and it can be seen that the order of activity as measured in mice by histamine challenge is in general agreement with that observed in children's protection.

And furthermore, in this series, the correlation is as good as that between field trial results and mouse protection test in mice.

For instance, vaccine v11 with a home exposure attack rate of 4 per cent comes first in the histamine test, the same as in the field results, first in histamine sensitization, second

in mouse protection.

Vaccine v17 with an attack rate of 8 per cent comes second in the field, second in the HSF test, third in the mouse protection test.

Vaccines v7, 6, and 5, the least satisfactory in the field, are also found the weakest by both methods.

Other vaccines give intermediate values. The vaccine third in the field is first according to the mouse test, fifth according to the histamine test, number four is right in between three and five, five between four and six, and so on.

Other investigators have observed that the mouse protective and histamine-sensitizing activities are not always parallel and that some treatments upon the substrate or upon the host may alter differently the two properties or the animal response, thus indicating possible denaturation or dissociation.

As for the separation of HSF from protective antigen as different entities, however, unsuccessful attempts have been reported recently by Munoz and Hestikin who used a refined method of electrophoresis.

The others tentatively concluded that the mouse protective antigen is similar and perhaps identical to the histamine-sensitizing substance which would be in agreement with the suggestion by Joo, et al., 1960, that they form perhaps common antigen complex.

For this paper, we have gathered some observations

on various pertussis fractions and their protective and histamine-sensitizing activities. Some of these fractions may be of interest either on account of the actual bibliographical context or as possible approaches to purification and fractionation.

Next slide, please. (Slide 2)

First, a history of various fractions, crude or purified, investigated, and that mostly on a semi-quantitative basis.

The plus or minus signs represent the qualitative interpretation of their protective and histamine-sensitizing activities.

As can be seen, the two properties are found together as strongly positive in the majority of cases with signs two plus right in the Hughes extract, for instance, and they may be weakly positive in others such as a dioxane extract and residues.

Occasionally, we have observed discrepancies, for example, with the ~~pyrodine~~ pyridine extract plus or minus in the case of protection and minus in the case of sensitization.

Such results could perhaps correlate ~~Dobby's~~ Dolby's report of partial separation of HSF from the protective antigen.

Another case could also be analogous to that of the urea, tri-urea and formalin residues which with Maitland was interpreted as possibly due to a difference in the threshold of detection of different biological effects rather than the

operation of two different entities.

Some of these particular cases are examined in more details in the following slides: (Slide 3)

Two organic solvents, dioxane and pyridine have been provided for extracting antigenic material from whole cells. Dioxane was diluted 40 per cent in water, pyridine 80 per cent. And it was used according to a modification of the first part in Labzoffsky's technique, 1959.

The histamine-sensitizing and protective properties of the fractions as well as those of the original cells have been investigated, and the results are given in terms of number of dead mice over number challenged and percent or number survivals over challenged.

In the case of dioxane fractions, the extracts show very little of both activities with 3 per cent and 11 per cent, whereas the residue is active, 34 per cent and 43 per cent.

With the pyridine fractions, HSF is nil and the protective potency has been reduced considerably, 23 and 16 per cent, especially if one considers the quantity of material injected up to 3.3 milligrams.

Further consideration could, therefore, be given to dioxane for the purpose of fractionat on, but pyridine should be counted as an additional agent which seems to affect the antigens.

Next slide, please. (Slide 4)

DNA protein fraction was prepared from B. pertussis cells and purified according to the method devised by Olitsky, et al., for B. abortus.

The DNA content of this fraction is 54.5 per cent or five times that of the original whole cells. One can see here that using high doses up to 1 and 5 milligrams protection is considerable with both the DNA protein and the residue with 72 and 92 per cent protection.

By contrast, similar high doses, 2 and 5 milligrams of the two fractions, give a very low histamine-sensitization response -- 3 and 10 per cent for the DNA protein and residue.

Therefore, here again, as in the case of the pyridine, there is an indication of partial dissociation of the activities.

Next slide, please. (Slide 5)

Bacteria were treated in the Mickle disintegrator and the cell walls were separated and purified according to the classic method of Salton and Horn. That is, two successive centrifugations at 3,000 and 14,000 rpm between each of 12 consecutive washings alternating with distilled water and saline.

Under our experimental conditions, the cell walls and the cytoplasmic fraction protects the animal against intracerebral infection, 37 up to 70 per cent for the dose indicated.

In the case of the histamine-sensitization, on the other hand, the mortality rates are rather low, but positive

for both fractions.

The HSF activity of these cell walls would seem to be intermediate between the results reported by Munoz, et al., 1959, and more recently those by Sutherland, 1963. It is not known whether this property is due to the cell walls themselves or supposed contaminants as suggested by the later article.

Next slide, please. (Slide 6)

Our cell walls derived from Mickle disintegration, the left-hand side microphotograph, compare well enough with electromicrographs of cell walls of pertussis and other bacteria found in the literature. Those on the left have been used in our biological experimentation.

We have also prepared cell walls from bacteria disintegrated in the ~~used~~/ Hughes press and in the Eppenbach-Coleman mill qv-6 model. The respective groups are shown in the center at the right.

It should be pointed out that such preparations were found impossible to purify to a satisfactory degree with the above-described techniques.

Your attention is called to these small particles on the cell walls which do not seem to be easily separable from the ~~used~~/ Hughes type cell walls.

Next slide, please. (Slide 7)

The two activities of the cell walls have also been shown in comparative passive tests in mice, using antisera

prepared in the rabbit. A schedule of hyperimmunization was the same with cell walls and the controlled whole cells.

The results of passive protection against intra-cerebral innoculation on the right-hand side are similar for both antisera and superior to those obtained with normal rabbit serum even at the dilution of one in 25. Passive protection tests are, therefore, in agreement with the active immunization.

For anti-HSF activity, different groups of mice received either the histamine challenge only or the sensitizing vaccine plus the challenge or the serum plus the sensitizing vaccine plus the challenge. The results are expressed in terms of deaths over number challenged for purposes of uniformity with active tests.

The results show that the anti-bacterial serum is potent since even at a dilution of one in 100 to one in 125, the proportion of dead is still less -- nine out of 25 -- than in the vaccine group -- 11 out of 14.

And it can be seen also that the anti-cell wall serum is found capable of protecting the animal against HSF. It is less effective, however, than the anti-bacterial serum for the three dilutions tested.

The presence of HSF in the cell wall preparations is nevertheless confirmed -- next slide, please (Slide 8) -- by phenol extraction of pertussis cells and cell walls according to the mthod of Westphal. We have prepared the lipopolysaccharides

which amongst other biological properties is known to be highly pyrogenic. This may refer to some observations made yesterday about the pyrogenicity of bacterial vaccines.

The biological properties of these preparations were reported in the past and we will limit ourselves here to HSF and protective antigen inactive immunization tests, the LPS, as well as the related fractions. The phenol phase and residue gave low survival rates from 5 to 15 per cent even at doses as high as 2.5 milligrams per animal.

LPS was, therefore, considered inactive, but our conclusions at the time did not rule out the possibility that when in the cell or the cell walls, it may play more than the role of nonspecific adjuvant mentioned by Fartung.

However, results are in agreement with Sutherland's who used the indirect method of passive protection tests with LPS adsorbed antibacterial and anti-cell wall serum.

In the HSF tests, on the other hand, there were 28 to 36 per cent deaths with LPS and 15 to 35 per cent for the related fractions. If these results were not due to product toxicity, it would be the first instance in our series of tests that the histamine-sensitization response is superior to protection on the same material weight basis.

Next slide, please. (Slide 9)

We will pass this one.

Next one, please. (Slide 10)

This last slide calls for comments on a later part of our experimentation. Differential centrifugation was used for fractionating disintegrates of B. pertussis cells treated in the Eppenbach-Coleman mill.

It was observed that the 100,000 g supernatant of the residue contains rather homogenous microscopic particles. These are illustrated by the electron micrograph on the left-hand side. The approximate size of these particles -- 30 to 40 millimicron -- is about the same as that of the small contaminants found in pertussis cell wall preparations from cells disintegrated in the ~~used~~ Hughes press illustrated top right.

The juxtaposition of pictures is not to infer that these particles are of cell wall origin. With an RNA content of 2.6 per cent and DNA 18 per cent, these could derive from the cytoplasm or the nucleus of the cell.

As shown at the right bottom, such particles are antigenic in vitro.

Diffusion tests against antipertussis serum show two or three precipitin lines with the fraction 100,000 g which is in q. L is the original cells and here is represented other differential centrifugation fractions.

Systematically, the protective and histamine-sensitizing properties of these fractions have been tested, and the preliminary results indicated that even in such submicroscopic particulate fractions of the cell, both activities can be detected

if very high doses are used. Five milligrams give four survivals out of 15 after intracerebral challenge. And following histamine challenge, there is only one death out of 20 with 4.5 milligrams and three out of ten for 13.5 milligrams

The isolation of particles from different microorganisms has been reported by other investigators, and in the case of _B_. pertussis, it is not known if they are analogous with the platelike material mentioned by Eldering, 1962, or the platelets of Millman, et al., 1962.

In resume, both the protective and histamine-sensitizing activities have generally been found in a variety of pertussis fractions as well as in bacterial vaccine. Two additional cases have been presented in which HSF seems to have been affected more readily, -- namely, the pyridine extract in residue and the purified DNA protein fraction.

Cell walls prepared and purified according to the method of Salton and Horn have shown both properties.

The HSF activity seems intermediate in comparison with the results obtained by other investigators. Furthermore, the presence of HSF and protective antigen in the cell walls was confirmed by anti-HSF and passive protection tests with cell walls antiserum.

Large doses of lipopolysaccharide did not protect mice. The HSF response may have been only apparent and due to toxicity. As observed in preliminary experiments, both the HSF and

protective activities could be detected even at the level of submicroscopic ~~particular~~ particulate fractions.

The need for purified protective pertussis antigen was emphasized at the Prague conference last year, and anyone concerned with this problem of purification has to cope with HSF.

The remarks of Preston and ~~Tapunga~~ Te Punga, 1959, may still apply. "There is no convincing evidence in the literature to show that HSF differs from the immunizing antigens, although there is as yet inadequate proof that they are the same."

Thank you.

(Applause.)

DR. ELDERING: Thank you, Dr. Guerault.

Our next paper reminds us that even before we have solved one problem, we can see beyond it further problems. Dr. Cohen will speak to us on this topic: Are the specifications for whole-bacterial vaccine adequate for fractions?

DR. COHEN: Dr. Eldering, Dr. Pittman, a number of years ago, we became interested in the method of Pillemer in obtaining protective antigen by ultrasonically destroying pertussis cells. We used 40 hours' old cultures from a fluid medium and treated them ultrasonically during 30 minutes.

We centrifuged the bacterial at 8,000 g, and we found that the resultant supernatant and the protective antigen therein would easily be adsorbed on ~~medium~~ aluminum phosphate. It

ORDER OF CLASSIFICATION

Vaccine No		V11	V17	V15	V12*	V8	V10	V14	V7	V6	V5
FIELD TRIALS	ATTACK RATE %	4	8	9	12	14	29	48	56	57	61
	CHILDREN'S PROTECTION	1	2	3	4	5	6	7	8	9	10
LAB. TESTS	AGGLUTININS in CHILDREN	2	7	1	3	4	5	6	8	8'	10
	AGGLUTININS in MICE	4	7	3	2	5	6	1	9	8	10
	MOUSE PROTECTION	2	3	1	5	6	7	4	8	9	10
	HISTAMINE ** SENSITIZATION	1	2	5	3	4	7	6	8	8'	8''

* Mean of 2 data.

** Deducted from Maitland, H. B. et al., J. Hyg., 53: 196-211, 1955 (Table 5).

All others calculated from Brit. M.R.C., B.M.J., i: 994-1000, 1959 (Table VII).

Table 2

COMPARATIVE ACTIVITIES IN MICE

TESTED	IcP	HS	TESTED	IcP	HS
B. pertussis culture	+	+	Cell walls	+	+
Bact. vaccine	+	+	Cytoplasm	±	±
Supernatant	+	+	Lipopolysaccharide	±	±
Cells	+	+	Related phenol fract.	±	±
Washed cells	+	+	Acetone extract	-	-
Washings	+	+	" residue	+	+
Hughes extract, residue	+	+	Dioxane extract, residue	±	±
TUF extract	+	+	Pyridine " "	±	-
" residue	+	-	Pepsin " "	+	+
DNA-protein	+	-	Trypsin " "	+	-
" " residue	+	±	Submicroscopic particles	?	?

(Table 3)

B. PERTUSSIS + ORGANIC SOLVENTS

	HISTAMINE-SENSITIZATION			INTRACEREBRAL PROTECTION		
	Dose (mg.)	Dead/ chall.	% dead	Dose (mg.)	Surv./ chall.	% surv.
WHOLE CELLS	0.003-0.5	21/39	54	0.003-0.5	97/136	71
DIOXANE EXTRACT	0.3-6.0	1/30	3	0.1-2.0	6/56	11
DIOXANE RESIDUE	0.03-0.8	13/38	34	0.03-0.8	44/103	43
PYRIDINE EXTRACT	0.2-2.2	0/30	0	0.2-3.0	16/69	23
PYRIDINE RESIDUE	0.03-3.3	0/48	0	0.03-3.3	14/85	16
Chall. controls	-	0/15	0	-	0/29	0

(Totals of 2 and 3 experiments respectively)

Slide 4

B. PERTUSSIS (DNA-PROTEIN)

	HISTAMINE-SENSITIZATION			INTRACEREBRAL PROTECTION		
	Dose (mg.)	Dead/ chall.	% dead	Dose (mg.)	Surv./ chall.	% surv.
WHOLE CELLS	0.01-0.1	23/40	58	0.01-0.1	26/30	87
DNA-PROTEIN	0.5-2.0	1/30	3	0.5-1.0	18/25	72
RESIDUE	0.1-5.0	3/30	10	0.1-5.0	24/26	92
Chall. controls	-	0/30	0	-	0/30	0

(Totals of 3 and 2 experiments respectively)

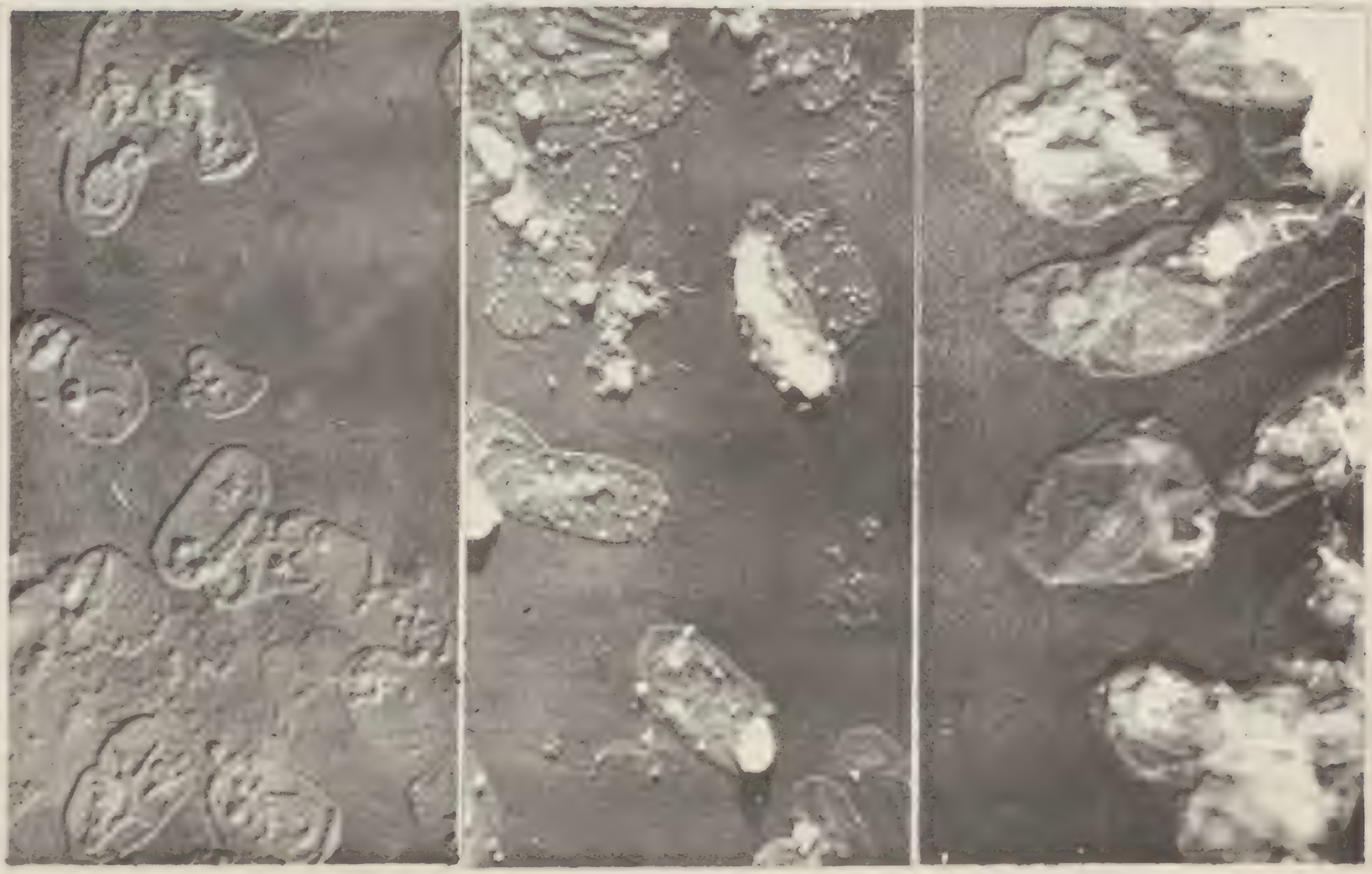
(Slide 5)

E. PERTUSSIS STRUCTURAL FRACTIONS

	HISTAMINE-SENSITIZATION			INTRACEREBRAL PROTECTION		
	Dose (mg.)	Dead/ chall.	% dead	Dose (mg.)	Surv. / chall.	% surv.
WHOLE CELLS	0.2	1/25	4	0.12	10/45	22
	0.8-1.2	19/50	38	0.6	24/46	52
	3.2-6.0	24/30	80	3.0	26/45	58
CELL WALLS	0.048	0/10	0	0.024	21/57	37
	0.24-0.48	2/38	5	0.12	33/58	57
	1.2-2.4	13/37	35	0.6	28/40	70
CYTO-PLASM	0.024	0/10	0	0.024	18/60	30
	0.12-0.24	3/40	8	0.12	23/60	38
	1.0-1.2	3/20	15	0.6	37/52	71
	4.8	3/10	30	-	-	-
Chall. controls	-	0/40	0	-	0/40	0

(Totals of 4 experiments each)

B. PERTUSSIS CELL WALLS



MICKLE HUGHES EPPENBACH

(Slide 7)

RABBIT SERA	ANTI-HSF ACTIVITY (Dead/chall.)			EFFECT ON I.C. INFECTION (Surv./chall.)		
ANTI- DIL.	1:1	1:5-10	1:100-125	1:1	1:5	1:25
WHOLE CELLS	3/25	13/24	9/25	30/36	22/36	5/36
CELL WALLS	12/35	26/35	24/34	28/36	19/36	7/35
NORMAL	9/14	-	-	5/36	2/36	2/36
Controls: Vacc. + chall.			11/14		-	
" : Chall. only			0/20		0/20	

(Totals of 2 experiments each).

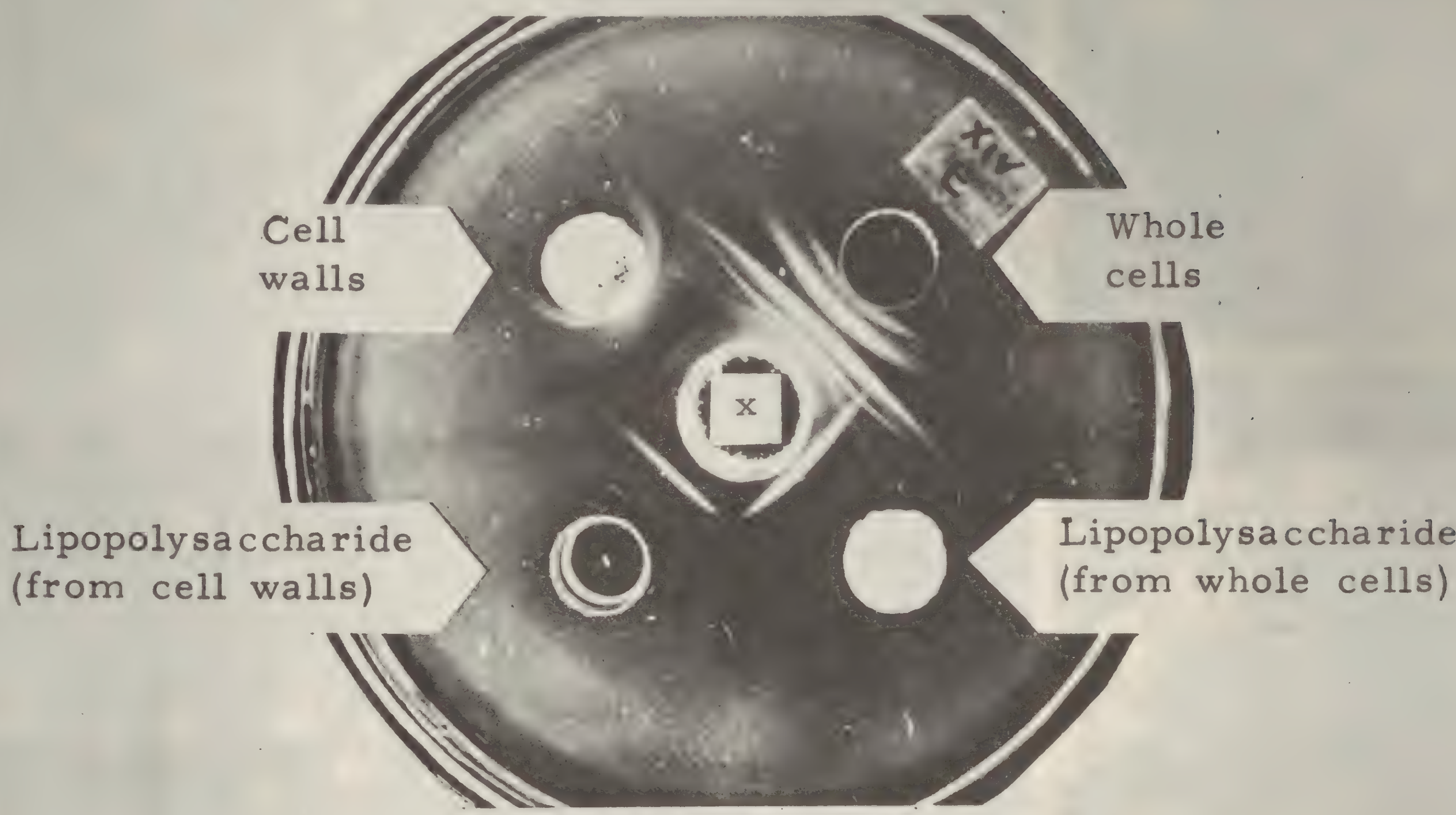
B. PERTUSSIS PHENOL EXTRACTION

	HISTAMINE-SENSITIZATION			INTRACEREBRAL PROTECTION		
	Dose (mg.)	Dead/ chall.	% dead	Dose (mg.)	Surv./ chall.	% surv.
WHOLE CELLS	0.016	6/30	20	0.016	20/46	43
	0.08	17/30	57	0.08	24/42	57
	0.4	20/30	66	0.4	23/34	68
LPS	0.1	11/39	28	0.1	3/56	5
	0.5	14/39	36	0.5	5/68	7
	-	-	-	1.0-2.5	2/16*	13
PHENOL PHASE	-	-	-	0.1	0/52	0
	0.5	6/40	15	0.5	4/51	8
	2.5	17/48	35	2.5	9/59	15
RESIDUE	-	-	-	0.1	2/51	4
	0.5	10/40	25	0.5	3/51	6
	2.5	10/47	21	2.5	3/65	5
Chall. controls		1/30	3	-	0/30	0

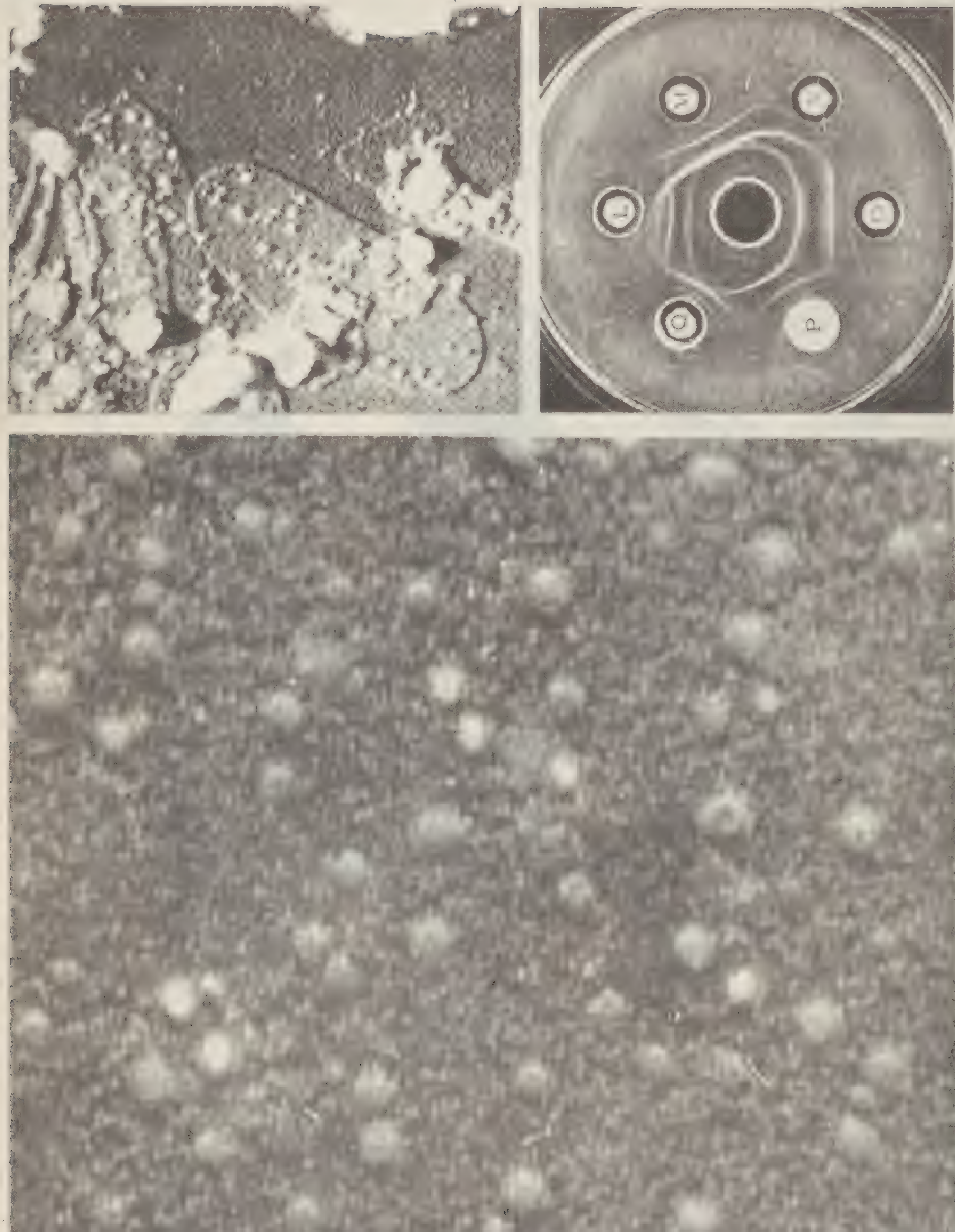
* Mortality before chall. $\geqslant$ 50% at doses $\geqslant$ 1 mg. LPS.
(Totals of 3 experiments each)

(Slide 9)

IMMUNODIFFUSION IN AGAR



x Whole cells antiserum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could be detoxified before by heating it 30 minutes at 56 degrees.

By treating 70 hours' old cultures, we found that there was a difference in the release of protective antigen and histamine-sensitizing factor.

May I have the first figure, please? (Figure 1)

Here is the time of treating the culture to 60 minutes, and here you see the yield in the supernatant of the protective antigen and histamine-sensitizing factor.

And actually, we decided to make a DPT preparation by adsorbing heated supernatant from 70 hours' old culture -- (Table 1) may I have the next slide? -- to combine with purified antigens and to compare the reaction in children with two DPT vaccines with intact organisms.

This is the potency of this preparation in 8 mg/ ~~opacity units~~, the total immunizing dose being 48 opacity units, so the total protection is six times as high as these.

The potency of three preparations was about the same as selected here.

You see that the survival for total after histamine challenge, after injection of six, 1.2 and 0.24 mg in mice, were different in these preparations.

There is virtually no histamine-sensitizing factor in this preparation, and in the next slide. (Table 2) Dr. Tasman in our institute compared in children the reactions after injections

of these three vaccines. The code means, (a) no reaction and observed within 24 hours; (b) is a local or slight local reaction with a temperature not exceeding 100 degrees after six hours, measuring it rectally. And (c) means somewhat larger slight general or general reactions of the child. And you can see from the results that virtually the purified pertussis component in the DPT and the DPT reacted in the same way in these children.

Perhaps it was a little bit less in babies and a little bit more in older children with this preparation, but we didn't think that these results led us to anything which was worthwhile to continue. And it actually had a big impact on our way of thinking.

After the completion of these experiments, we decided to try to obtain soluble protective antigens, and we wanted to pay attention to the complete separation of the different toxic factors and the protective antigen, HSF included.

I think Mr. van Hermet and Mr. Van Wezel in our laboratories succeeded by using extraction of sodium desoxycholate. And may I have the next slide?

We used the culture fluid for continuous cultures. I will show you some results later on. We obtained a bacterium by acid precipitation, and we got a cell suspension in this way which was treated with desoxycholate followed by DNA-ase and centrifugation. ~~centrifuge.~~ We got an antigen solution. We applied gel

filtration over Sephadex 200 and followed -- but we are not always successful at this point -- by serum filtration.

And in the next slide, I can show you some of the results we got.

This is the continuous culture, 1:13. It means that this is a mixture of samples taken of the whole bacterial mass -- the code is here -- whole bacterial mass taken from 1:13 and per doses, so you have to multiply in this case by three to get the total immunizing doses with the protective antigen. And here you get extractions on three different days which had 4.2 protection, 1.4 and 4.0.

The next experiment is about the same. 7.2 in the starting material, 3.6, 6.4, 6.2.

And here we have the histamine-sensitizing factor which we express also in units, using the method of Preston and comparing it with our Dutch reference preparation for the potency test.

So actually, we should expect these values to be the same, and I will come back to this later on.

Here (indicating) you see a number of other experiments. This is the original bacterial mass again from experiment 127. Here you see the extractions from culture number 8, 15, 15 -- this is all the same -- and here is a final experiment.

This is a very high value, of course. And here you see the HSF values.

(Figure 2)

But I wanted to give you in the next slide/ the ratio between the mouse protection activity and HSF. We expect a value of -- this is 10, this is one, this is 0.1 here. So you see the original bacterial mass, this ratio expressed between 10 and 0.1. But we can easily imagine that the average is about one.

The acid precipitated material perhaps is a little bit lower, but we have the definite feeling that the ratio mouse protection HSF is lowest in the solubilized material. This could be explained in two ways.

The first point, the first explanation, could be that during the solubilization process, there is a high release from the bacterial cell of HSF, both factors being separated during the process.

But there is also another explanation, and that is that mice are reacting biologically different to a solubilized HSF as compared with an HSF component which is prevalent in the cell wall.

Anyhow, these results in which we obtained these relatively large HSF values would certainly withhold us from experimentation in children. We want, moreover, to have more information about the protective antigen and how it will immunize children. Especially what we heard yesterday about the duration of immunity in children from Dr. Eldering should make us very cautious to proceed with a soluble immune antigen

from which we do not know more than that it protects mice. And I think the same is true for the toxic factors.

Solubilization is the first step, and it should make possible first a purification of the different toxic factors. And it should be possible to measure their impact on the micro-organism and to measure the pharmacological activity. And it might be possible that eventually new requirements for solubilized and purified protective antigen will be needed.

Thank you very much.

(Applause.)

DR. ELDERING: I believe this concludes the formal papers, and now the meeting is open for discussion. Dr. Brown will open the discussion for us.

These papers should really stimulate a lot of interesting comments.

Dr. Brown.

DR. BROWN: Dr. Eldering: I think from the pre-eminence of the principal organizer of this meeting and the pre-eminence of the outstanding workers in pertussis, it would be appropriate to say that we have opened these last two days a Pandora's box. We found a lot of surprises. We found some new things. We have rehashed some old points of contention. I expect we will rehash some more tomorrow.

(Laughter.)

But in thinking about it, one wonders what all of

table 1

Release of protective antigen and histamine sensitizing factor after supersonic treatment of living B.pertussis bacteria

VACCINE Nº	POTENCY (PU-8000 mg)	SURVIVALS / TOTAL AFTER HISTAMINE CHALLENGE 6000 mg	1200 mg	240 mg
B 1 (PURIFIED ANTIGEN)	2,1	19/20	18/20	20/20
38 (INTACT ORGANISMS)	1,8	5/20	17/20	18/20
47 (INTACT ORGANISMS)	1,8	12/20	19/20	19/20
			C = 20/20	

figure 1

Potency and H.S. properties of 3 D.P.T. vaccins. The toxic properties of which were also compared in children

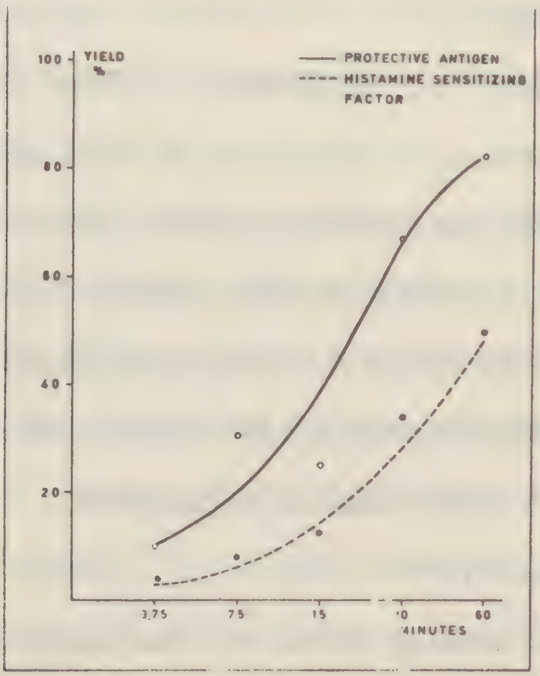


figure 2

Decrease in the ratio M.P./H.S.F. after solu bilization with sodium-desoxycholate of acid precipitated bacterial concentrates

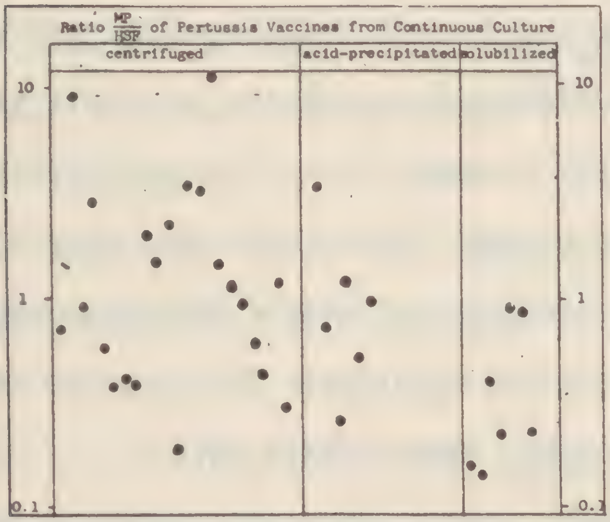


table 2

Toxic reactions in children of different age-groups 24 hours after injection of a DPT vaccine with a purified pertussis component and two control DPT preparations

CONTROL VACCINE	CATEGORY	0-1 YEAR OLD	1-3 YEAR OLD	3-6 YEAR OLD
DPT	A	80	13	25
	B	27	6	18
	C	8		
PURIFIED DPT	A	109	16	20
	B	16	8	23
	C	6	1	1

A = NO REACTION
B = LOCAL OR SLIGHT LOCAL REACTIONS
C = SLIGHT GENERAL OR GENERAL REACTIONS

this information means in that I believe only two of the workers -- Dr. Munoz and Mr. Levine -- mentioned strains in their discussion. I think anybody that has worked with pertussis is aware of the fact that the strains vary widely in their response to different treatments and their response antigenically and their response from a toxic viewpoint.

We had this brought out to ourselves some 15 or more years ago in sonic extraction to recover endotoxin where we found some strains released relatively little or no toxin whereas other strains gave several thousandfold more.

In connection with Dr. Wardlaw's paper on sonic extraction, I would like to comment that we do use this method in large scale to produce toxin to immunize rabbits and found that the endotoxin was released with 30 to 35 minutes of extraction at 9 KC that apparently it was a true toxin in that it could be detoxified, converted to a toxoid with formalin, and that it was antigenic when used to hyperimmunize rabbits.

It also apparently did carry with it some of the histamine factor in that the hyperimmunization had to be carefully done in order not to kill the rabbits.

Mr. Levine's paper is quite refreshing. So often people don't have the courage to stand up and report their negative results and failures. I think it is quite interesting that he in his work did cover, I believe, ten strains of the pertussis organism.

MR. LEVINE: Six strains.

DR. BROWN: Six strains.

As for the other papers, I don't consider myself in a position to comment on them, and I think probably it would be best to hear from the floor on these.

Dr. Eldering.

DR. ELDERING: Do we have people who would like to open up further discussion at this point?

DR. PITTMAN: Dr. Eldering, you did some of the very earliest work on fractionation of the antigens of pertussis, and you have continued to work in this field. You have done so recently. I wonder if you don't have some comments to make.

In fact, I tried to get her to give a paper, and she refused. But I thought maybe she would have some slides in her pocket she could show to us.

DR. ELDERING: Compared to some of the work such as that reported by Dr. Munoz, mine was like a Model T compared to a Cadillac, something like that.

I think that the experiences with purifying antigens from pertussis in all the different laboratories have been very interesting in that so many times someone has a successful effort which not only is not repeated when someone else tries to repeat it in another laboratory, but also the original workers are unable to repeat it. So that the critical factor so frequently is not known.

I remember at one time we were so excited because we had sonically disintegrated culture which we had treated with alcohol. I have forgotten the details -- 10 per cent. And we had good mouse protection in this fraction which we were able to repeat. We were unable to produce agglutinins in mice, and we thought everything was just fine. And we were never able to repeat those experiments.

Fortunately, we didn't publish that, and I am not publishing it now. I am just giving it as an illustration.

(Laughter.)

Recently, we have gone on with sonic extraction because it is the only method we have of disintegrating the organism. And we no longer think we are searching for a soluble antigen by the methods that we are using. We really have a particulate antigen which we are able to use for mouse protection tests and get reproducible results with this particular material which we believe are cell walls.

Now, of course, Dr. Munoz has gone further and has extracted these and has a soluble antigen which is wonderful. And I think that's the next step. He has means beyond ours in our little laboratory to use.

I think other people have to go on from here. But surely we have some people with -- I am not going to show a slide even if I have one in each pocket. I think some other people should discuss this now who have really done much more than we

have in Grand Rapids with this part of the work..

DR. MUNOZ: May I just --

DR. ELDERING: Yes, Dr. Munoz.

DR. MUNOZ: I am always extremely impressed by Dr. Eldering's modesty. I consider her one of the authorities in pertussis and have followed her work very closely. And I think she has been through as much as anyone in this field.

You shouldn't feel so modest about it.

One of the comments that I have to make -- this is with respect to the relationship between the protection or protective antigen and histamine-sensitizing factor .

As all of you know, I have stuck my neck out and said that they are possibly identical. The evidence presented here shows that maybe they aren't identical.

Now, this is what I want to make comments on because the persons are trying to compare the protective activity and the histamine-sensitizing factor on two tests that are completely different. And let me point out where the things differ.

The HSF is a very short-time test. You can demonstrate HSF activity one day after you inject this material and probably even sooner. But routinely, one does it at four days -- at least we do. The protective activity is measured two weeks later. Adjuvants do not affect in the least the histamine-sensitizing activity. As all of you know, adjuvants will greatly increase protective activity.

In spite of what has been published, in our hands the HSF is not neutralized by antibody if it is in solution. This is a very peculiar thing, but in solution, we cannot neutralize HSF. It is neutralized when one uses whole cells.

The insinuation here to me is that the neutralizing substance probably doesn't have anything to do with reacting with HSF. Or, if it does, the reaction that takes place does not neutralize the activity.

The fourth point that I want to make is that the HSF activity is affected markedly by the route of immunization or injection of this substance. The protective activity probably is also affected, but not as markedly.

Subcutaneous injections of HSF, for example, have very little or show very little activity.

The fifth point that I want to make is that the mouse strain that is used is extremely important and most mice, most strains, do not sensitize to histamine, but they protect against pertussis challenge.

And I think this point of the mouse strains should be emphasized because no one mentioned what strain of mouse they are using. And this, to me, is critical.

The other point is that even environmental conditions greatly affect the histamine-sensitizing test, HSF test. If the mouse is stressed in any way, they will not sensitize as well. And minute changes sometimes affect this test. And

if the conditions are not ideal in our hands, we get tremendous variability.

In some cases, and this also happens in the protection test, but not as marked, you can have two identical jars with mice. In one jar, five mice will survive and the other one five or four will die after challenge with histamine.

Now, what is the difference? This is highly statistically significant.

We have observed this from time to time that we were working with Lee Schuchardt at Merck --

MR. SCHUCHARDT: Not that old, Jack.

(Laughter.)

DR. MUNOZ: So what?

But these points I think should always be kept in mind in comparing the histamine-sensitizing activity and the protective activity. And if you don't pay attention to these things, you are going to get all kinds of results and answers that will confuse the issue.

I may say again that in our hand fractions that contain one main antigen by agar diffusion, and I have to say that the agar diffusion test is not the final proof of purity -- in fact, it is a very crude proof of purity. If you don't have the antibody for the substance you are measuring, you are sunk. You cannot detect anything but whatever you have antibodies for

But we can eliminate from 12 antigens that I am sure

of -- our antiserum has perhaps more -- we can eliminate eleven of these antigens, and we end up with a fraction that has both histamine and protective activities and that anything we have done, which is mild, will destroy or affect the two properties identically.

This is what we base our conclusion that the two might be in the same molecule.

DR. ELDERING: Dr. Munoz, you are getting down to the molecule now, and you are saying that the antiserum against the protective antigen will not neutralize the histamine-sensitizing factor.

DR. MUNOZ: That's right.

DR. ELDERING: I can't see that molecule. I want some help.

DR. MUNOZ: Well, there is a very close similarity to this in enzymes. You can prepare ~~compare~~ an antibody to an enzyme, precipitate the enzyme, but the activity remains unaffected. And this would be similar to that.

DR. ELDERING: All right, I am beginning to see it, just beginning.

Dr. Perkins.

DR. PERKINS: I think that one of the few things that have made an advance in our extract pertussis work is Pillemer's material. This is the only material that I am aware of that is being subjected to a very good clinical trial, a

protection trial.

Undoubtedly, Pillemer extracted a very small fraction of the organism. He was mainly on long lines when he said if it was a protein, then it was less than 1 per cent of the organism because he couldn't detect any nitrogen decrease in the material after he had adsorbed onto these red cell stromata the protective antigen. And this material is very strongly histamine-sensitizing, produces tremendous quantities of agglutinins in children, in rabbits, in mice, and in the child produces as many reactions as whole bacterial vaccines.

And I just wondered whether in our approaches, we are not going too far in trying to dissociate all these things. We are after an extract vaccine that removes the majority of the protein which we can call extraneous protein that we don't want to innoculate into children. But at the same time, we are not protecting mice. We are trying to protect children.

And I think if we can get an elegant method for disrupting these organisms and extracting out the material that is going to protect them, if it does happen to have HSF, if it does happen to have agglutinin, again, so what, as long as it protects the children. And I think this is what we must aim at.

DR. ELDERING: It would be helpful, though, if we knew the whole truth, wouldn't it?

DR. PERKINS: Yes, but, unfortunately, we have got to somehow decide what our criteria are to be before it is to

be subjected to a protection trial.

DR. ELDERING: We are after parts of the peel of the little pertussis bacillus, and I don't know whether I am correct about this or not, but in Pillemer's antigen, he had ~~distributed~~ disrupted these antigens sonically. He must have pieces of the cell wall. Those were attracted to the autoclaved red cell stromata. They were attached to it, but they must have been pieces of cell wall. Isn't that correct?

DR. PERKINS: I don't think there is any doubt about it.

DR. ELDERING: But the cytoplasm containing most of the protein and what is probably left.

DR. PERKINS: Yes.

DR. ELDERING: But he himself said this was not a purified material.

DR. MILLMAN: I don't believe Pillemer separated the cytoplasm from the wall after disruption, did he? He added the stroma to it.

DR. ELDERING: Only by the process of selected adsorption by the red cell stroma.

DR.MILLMAN: The stroma could have adsorbed --

DR. ELDERING: Could have -- I don't know whether it did or not. It must have to be so toxic as it was, I suppose.

Who else would like to say something now?

Yes.

MR. SCHUCHARDT: I have a question for Dr. Munoz.

He presented a very nice table where he showed his ultracentrifuge data, and he gave the results for protective antigen. I wonder if he has the same results for the histamine-sensitizing activity for these same fractions and also whether or not this ~~would~~ was run in a ~~comparative~~ preparative centrifuge and/or analytical, and if it was analytical, what his peak looked like.

DR. MUNOZ: The histamine-sensitization are identical as far as I am concerned. In fact, as I mentioned, I could change the title of the talk and talk about the HSF and tell you exactly the same thing that I told you.

The ultracentrifugation studies were done mainly in the ~~comparative~~ preparative. We have done also studies on the analytical ultracentrifuge. And in there, you get a very smeary looking picture. There is something that sediments very quickly which is probably fragments of cell walls. And then you get an unusual peak which very quickly starts to disintegrate more or less.

This is one of the evidences that I have for saying that the material is ~~partly dispersed~~ polydispersed, but these studies are preliminary, and I wouldn't want to make any statement other than that.

In fact, I am always afraid of the girl taking notes here.

(Laughter.)

DR. ELDERING: Dr. Perkins.

DR. PERKINS: Dr. Munoz touched on the point of the

mouse strain. This is really a most important point, and at the Prague conference, I recall we almost decided there should be a strain shipped throughout the world for our work.

We did this many, many years ago with the Hartley guinea pig strain for trying to get some sense out of diphtheria work. And I wonder whether Dr. Munoz would agree this was such an important part of our future progress, whether he would agree that we should start investigating in our different laboratories one or two strains of mice that should be eventually adopted for this work to get some uniformity throughout the different countries in the world.

DR. MUNOZ: I would definitely agree with what you said. The unfortunate part of it is that you cannot get a uniform mouse strain as soon as you ship it to another laboratory.

Our original observations on the mouse strain were done on two different lines of the same strain. In fact, Lee Schuchardt was the one that ~~make~~ made the observation. The mouse that Sharp & Dohme at that time had was sensitive to the action of the HSF, and they ran into some complications of Salmonella infection or something like that. So they had to start all over again with a small nucleus of the same strain.

The strain that they ended up with was not sensitive to ~~merthiolate~~ histamine. So this is an extremely touchy problem.

I have had very good luck with the CFW strain, and I was unfortunately accused by a very well recognized authority

in the pertussis histamine sensitization work of erroneously calling this strain nonsensitive while, in fact, what I was calling insensitive was a CF-1. And they are both from the same commercial house. And the CF-1 strain is totally insensitive in our hands when you test it at a 14 to 20 gram level. That is the mouse weight. If you wait longer than that, I don't know what will happen.

Dr. ~~Profenjev~~ Parfentjev has shown that old mice become sensitive without pertussis to histamine, but this is a very complicated problem and you have to standardize your mouse. There is no question about it. But you cannot insure that all the laboratories in the world will have the same mouse strain as soon as you start shipping this colony out.

DR. ELDERING: Dr. Pittman, do you have --

DR. PITTMAN: I would like to make a few comments about mice that are capable of being sensitized to histamine in relation to their ability to be protected. And we have found that the mouse that is the most capable of being sensitized to histamine is the best immunizer. And the CFW mouse is the best of all that we have studied, not only for immunization against pertussis, but tetanus and diphtheria. So it is a good tool in selecting whether you have a good immunizing mouse.

This was observed by Dr. Csizmas in my laboratory several years ago.

DR. ELDERING: Yes.

MR. SCHUCHARDT: Lee Schuchardt, Merck, Sharp & Dohme.

I would like to add one more word to that, Dr. Pittman. CFW is one of the poorest for toxicities with a very slow --

DR. PITTMAN: Separate test.

DR. ELDERING: Dr. Guerault.

DR. GUERAULT: I should like to comment on strains, both bacterial strains and strains of mice used for protection or histamine sensitization.

I had to shorten my text and, therefore, I gave up some precisions for these tests.

Now, the greater part of the work to be presented has been done years ago. And ever since, we have replaced bacterial strains, but we have not been able to replace mouse strains on a practical basis.

We use a substrain of CF-1, 22 to 26 gram. We know by experience also that the results are lower than with other mouse strains.

But, on the other hand, they give reproducible results if you keep the host factor weight under control. We had the same experience in England with the Schofield strain.

Now, perhaps if it is not possible to uniform strain of mice from one laboratory to another, perhaps one could use a comparison as reference although it is known that even for protective antigen, the ratio of an unknown to a reference varies with the strain.

That's according to the work of Dr. Edsall, I believe. I believe that's about all for now.

DR. ELDERING: Thank you.

Yes, Dr. Millman.

DR. MILLMAN: I would like to make a few comments particularly with reference to a paper we gave in Prague dealing with the subject that Mr. Levine has done and mostly negative results.

And actually, in terms of many of the papers that are in the literature, they were not negative results. They were positive results, but quantitatively negative. And I have found in reviewing a lot of this work and in trying to repeat and sometimes getting positive results and sometimes getting negative results that too often there is confusion dealing with the quantitative aspects of things.

For example, if we analyze Dr. Munoz' work, we find that his best fraction taken right off the starch block after electrophoresis LD-50 in terms of weight is identical to the starting material. So, in effect, he has got a purer fraction, there is no doubt about it.

Perhaps it is single antigen in terms of starting material as SE composed of 12. But what actually happened in eliminating the other 11 are we are dealing here with a fraction composed of 90 per cent inert material, 10 per cent active, or actually is the material dispersed elsewhere where

we can never find it.

Certainly, in terms of weight, there is no gain in specific activities.

With respect to Dr. Guerault's reporting work today, some of his fractions contain protective activity, certainly. How much emphasis was made on adsorbed material in spinning out some of these fractions? Were we to add charcoal or bentonite particles and other things into some of these sonicates, would we also pull out protective activity? Could we rightfully claim there is activity there?

What I would like to stress is something which we did try to stress strongly and recommend strongly in this Prague paper was that we should strive at some quantitation. We should indicate the total amount of activity or yield in any particular fraction, not so much in terms of a reference sample, for example, as is one twice as good as a standard reference. What was the starting material of the unknown? Did the starting material of the unknown contain 40 protective units per mill?

And what we are isolating is something which is two times an NIH reference, which is 16. That's far from 100 per cent yield or 50 per cent yield.

These things are very often forgotten or one assumes that there is protective activity and, therefore, that is it. Unless we actually, as Mr. Levine brought out very adequately

today, unless we can actually relate the activity we finally isolate in terms of what we start with, we can never really know where we stand in a fractionation program or how far we are getting in isolating a purified fraction.

DR. ELDERING: Thank you, Dr. Millman.

I think Dr. Millman has made a very good point. I think one thing we probably need to do is define our objectives. And in this room here, we have people with many different objectives, so who wishes to prepare a better immunizing agent against pertussis, and they don't care what the chemical composition of it is just so they get fewer reactions and better results, and others who are bent on pure research. And they want to know what this material is. They are not so concerned with the yield. And they probably realize that they may end up with something which won't work so well.

We may have to add another adjuvant or something to it to make it work in children, but we want to know what the nature of this material is.

DR. MILLMAN: Dr. Eldering, with reference to that point, I forgot, with many of the papers, we did not hear whether the adjuvant was added to many of the fractions that were isolated. Were they ~~aluminate and~~ aluminum phosphate adsorbed and such?

DR. MUNOZ: I should make a response to Dr. Millman's comments because I think he mentioned PD-50, not LD-50.

DR. MILLMAN: I am sorry.

DR. MUNOZ: If it is LD-50, our results don't gibe at all, but PD-50, they do. That is protective dose. Fifty is probably very close to the original bacterium on the weight basis.

DR. MILLMAN: On the weight basis, they are about identical.

DR. MUNOZ: Yes.

The other thing that I should mention is that we never use adjuvants. We use the straight material in saline or phosphate buffer, and this, in essence, might be the crux of the whole thing.

I have injected mice with 4 milligrams of egg albumin in saline, and they do not respond at all with antibodies to egg albumin.

I can give one gamma of egg albumin, one gamma in adjuvants, and they respond with antibodies. So this adjuvant effect is extremely important, and pertussis has an adjuvant effect.

We have not pinned it down to the protective activity or fraction, but if it is not, you can readily see that if you are just comparing activity without any adjuvant, you are going to reduce it by purifying it.

But I am not absolutely sure that the protective antigen as we have it now does not have the adjuvant effect.

We are doing work along this line.

DR. MILLMAN: Jack, have you tried your purified fraction as an alum precipitate?

DR. MUNOZ: No.

DR. ELDERING: Yes.

DR. REPENTIGNY: I would just like to make a comment on the analysis of the material, you know, and Dr. Munoz used the ultracentrifuge. But it would be very nice to see if you can have a picture under the electron microscope because all those fractions with high speed centrifugation, to have some kind of a structure, they should be visible with this, maybe some kind of a form.

If the destructive material is not homogeneous enough, maybe it is difficult as you said to put the finger on it with the ultracentrifuge, you know.

Like they do in virus work, you know, they always go together with the ultracentrifuge and electron microscope. Maybe it could be useful; I don't know.

DR. MUNOZ: I should make it absolutely clear that I am not positive that our material doesn't aggregate again to form structures that may be similar to what Ribi has observed with Pasteurella, I think, in which he solubilizes the cell wall. And this cell wall material comes back together into plates or fibers that obviously would be seen as particles under the electron microscope.

And I am almost certain that if we made electron microscope pictures of our preparations, we will see something. It will not be just like a film of small molecules because I think this aggregates.

All we have to do is freeze this material a couple of times and you get a precipitation. If you lower the pH slightly, below five, it precipitates. And during the drying procedure, I am sure that some of this material will come together, and you may see it displaced. But we have not done this. Dr. Malmgren has done it with endotoxin in which they found very, very little fibrils under the electron microscope.

So this is a possibility.

DR. ELDERING: Yes, Dr. Millman.

DR. MILLMAN: I just have one more comment with reference to what Dr. Perkins says about Pillemer antigen as being the very famous one and being on clinical trials.

I wonder if we shouldn't have some comment from some of the Lilly people who might be here who have also done some extensive clinical trials with solubilized protective activity.

DR. ELDERING: Is there someone here from Lilly who would like to comment?

DR. DETTWILER: Our clinical people covered that pretty well yesterday, I believe.

Dr. Peck, is that right?

DR. PECK: Yes, I thought so.

(Laughter.)

DR. MILLMAN: I was referring to some chemical data.

(Laughter.)

DR. PECK: I am a clinician, not a chemist.

DR. ~~DANIELSON~~ DETTWILER: I guess we don't have a chemist with us today.

DR. PECK: The only thing I have with me today that I didn't present yesterday is some data on the use of the modified neutralization test in comparing whole cell vaccines to the antibody response by the extrapolation.

DR. ELDERING: A slide in your pocket?

DR. PECK: I just happen to have one.

(Laughter.)

But we don't have a projectionist so I will read it off.

I think I mentioned yesterday about agglutinin response. If you divide the children, and I am talking here about several hundred children in each group, if you divide these children into whether they were less than three months of age or greater than three months of age at the time that immunization was begun, we found that with whole cell vaccines, as all precipitated DPT provides 85 per cent positive agglutinin response with extracted antigen 97 per cent. These two figures, if one wants to do a chi square, are the same.

In children greater than three months, when immunization

was begun 100 per cent with the whole cell vaccine, 93.5 per cent ~~were~~ with extracted pertussis. These two figures are also the same.

However, with the modified Munoz protection tests, same way, less than three months, greater than three months, we find that approximately 55 per cent of the children -- these are 78 children, by the way -- who received whole cell vaccine no matter what age, they were 55 per cent converted by this. I mean, had an increase, had a fold increase of at least twofold, or at least onefold in their antibody titer. With extracted antigen, 95 per cent showed an increase in antibody titer.

If one divides this as to whether they were less than three months of age or greater than three months of age, the significance is even greater with whole cell vaccines in the children that were six weeks of age when immunization was begun.

Fifty-four per cent showed an increase in protective effect after immunization whereas with the extracted antigen, 97 per cent showed increase.

This is the data I have.

DR. ELDERING: Thank you.

I wonder if Miss Mason from Ottawa who has done some work with fractionation would like to comment.

MISS MASON: I am afraid I am in somewhat the same position as Mr. Levine. I could write a book on the things I

can't do.

No, I would hesitate to comment any more than to say that so far, we have not succeeded in getting soluble fractions.

DR. ELDERING: Dr. Pittman, do you want to add anything to this? Are we finished?

Dr. Wardlaw.

DR. WARDLAW: Just one point I would like to make is this, that we know very little about the structure of gram negative cell walls, and the people who are studying this are working with coli, and there is some information available that the cell wall is a layered structure, sort of three-layered sandwich.

With another gram negative organism, *Spirillum* serpens, it is possible to show that the surface of the cell wall is a mosaic of macro molecules. This is studied with the electron microscope, and I would like to put out a plea that the same sort of thing should be done with pertussis to see if we can get some more fundamental information about the starting material from which we hope to extract the antigen.

We are in the position of someone who wants to isolate a serum protein without even knowing what the composition of serum is, and I think it would be very useful if we had some more basic information on the chemistry of the pertussis cell wall.

DR. ELDERING: Don't peel too thick.

DR. WARDLAW: And I think I am right in saying there

isn't a single gram negative cell wall protein from any organism that has been isolated in a pure state. I think the nearest that has been got to this is the complete "O" antigen, which is extracted from shigella, for example, or Salmonella by trichloracetic acid where you have a micromolecular complex of lipopolysaccharide protein and phospholipid, but I don't think even there it has been possible to get the protein component in the pure state.

DR. ELDERING: Thank you, Dr. Wardlaw.

I want to ask the most elementary question possible of Dr. Munoz.

Why do you use acetone extracted cells?

I could ask him afterwards, but I just want to show you how little I know about this.

DR. MUNOZ: This is an old method of --

DR. ELDERING: I know it -- 1940, before that.

DR. MUNOZ: I don't know how long ago ~~Landie~~ Landy applied it to extract the VI antigen.

DR. ELDERING: I used to have a little tube of acetone extracted pertussis in my desk for years and years. I finally threw it out. But why?

(Laughter.)

DR. MUNOZ: This goes back to Sharp & Dohme again. Sharp & Dohme by itself is dead, but Merck, Sharp & Dohme now.

(Laughter.)

When we were there, we were trying all kinds of fractionation, including the sodium desoxycholate, the acetone drying of cells and extracting it with saline. And at that time, we knew that this method was very efficient in extracting the histamine-sensitizing factor.

When I moved from Merck, Sharp & Dohme to Montana, I did not have the facilities to conduct sensitive mouse protection tests and made the decision of concentrating on histamine-sensitizing factor. And this is why I went back to that method.

And when I finally left Montana State University to the NIH, I had again facilities to conduct extensive protective tests. And every fraction that we had obtained at Montana State University which had histamine-sensitizing factor had also protective activity. And there is where I --

DR. ELDERING: But what does the acetone do? Does it break up some of the links in the cell wall and allow some of this material to escape? You are extracting lipid.

DR. MUNOZ: This is the most probable explanation that the extraction of certain lipid materials from the cell allow the protective antigen or histamine-sensitizing factor, if you want to call it that way, to be released.

DR. ELDERING: Does someone have a high note to close this on?

Dr. Culbertson.

DR. CULBERTSON: Dr. Millman used acetone in the extraction of the central antigen in the typhoid organism. That was the first use of this. This is the first time I heard of it.

DR. ELDERING: This was a long time ago.

Anyone have a high note to close this on?

Dr. Millman, I think you are the one.

DR. MILLMAN: If it will make you feel any happier, we repeat Dr. Munoz' procedures without the addition of acetone. The material is so toxic, you eliminate all your mice.

(Laughter.)

Acetone may also be very effective in perhaps allowing some lytic enzyme during the process Dr. Munoz has described in effecting the extraction.

DR. ELDERING: Dr. Culbertson.

DR. CULBERTSON: We need some germ-free mice tests.

DR. ELDERING: That's a good idea.

I guess we will adjourn now, Dr. Pittman. Is that all right?

DR. PITTMAN: That's all right. Be back at 1:30.

(Whereupon, at 11:40 o'clock a.m., the meeting recessed, to reconvene at 1:30 the same day.)

AFTERNOON SESSION

(1:30 p.m.)

DR. MURRAY: Can we get started, please?

My main function seems to be to quiet the audience until the chairman can take control, but it is now thirty seconds past the stated time, so we can get going.

We have been doing very well. We have rarely had a meeting that has been so punctual.

I would like to suggest in the interests of obtaining an improved record that those of you who have to leave early, but have slides, leave them with us for processing, and we promise to get them back to you intact within a day or so.

Those of you who have with you typewritten or photographic copies of the material on the slides, if you would give us those, we could make copies which would be much superior to copying the slides. We have had some pretty good luck thus far, but I regret to say these blue slides, while they are easy on the eyes, our machines are blind to them.

Dr. Wilson, carry on, please.

DR. WILSON: Thank you.

Dr. Murray, Dr. Pittman, I would repeat what Dr. Murray said as regards time. We have been very efficient in adhering to time so far in this meeting, and I would trust that the team this afternoon wouldn't fault. They have an enviable record to meet.

This afternoon's proceedings concern the stability of pertussis vaccine. A good deal of this will be concerned with preservatives, and I am reminded by something that I picked up yesterday, Dr. Murray, this is indeed a timely subject.

This is from a bag of peanuts, and it talks about four or five different preservatives being added. And underneath it says, "Record of the week: Check dial or record desired: popular, rhythm, country-western."

(Laughter.)

It is true; it is written right there.

(Laughter.)

DR. MURRAY: An unexpected bonus.

DR. WILSON: The first paper to be presented by Dr. J. M. Corkill from Canada is, "Influence of Preservatives on the Stability of Pertussis Vaccine, Alone and in Combined Antigens."

DR. CORKILL: Mr. Chairman, ladies and gentlemen: I hope that I am not the tuning fork that sets the note for the afternoon, but since 1948, Bordetella pertussis vaccine combined with diphtheria and tetanus toxoids has been extensively used for immunizing infants and primary school children in Canada.

In this combined antigen commonly known as DPT, a one in 20,000 dilution of merthiolate has proved to be an effective preservative. And experience has shown that the component antigens of this preparation are stable when stored for long periods of time in 4 degrees Centigrade.

In 1959, this product was largely replaced in our public health program by one in which the DPT was combined with poliomyelitis vaccine.

Our past experience may have given us a false impression that the DPT components were remarkably stable and, therefore, in the new product, the stability of the antigenicity of the poliomyelitis vaccine was the prime concern. Any changes required in the process of production could only be effected in the DPT fraction.

In our DPT, the pertussis vaccine was used as diluent for concentrated diphtheria and tetanus toxoids.

In the DPT plus poliomyelitis vaccine, it was necessary to concentrate the pertussis as well as the toxoids. And the poliomyelitis vaccine became the diluent.

Because the poliomyelitis vaccine was not stable in the presence of merthiolate, it was necessary to find other preservatives in which it was stable or else distribute the product in ampules and rely on the residual antibiotics of the poliomyelitis vaccine to control any infection occurring in subsequent stages of production and use.

For a short time, the new quadruple antigen was distributed in ampules with no added preservatives, but as distribution of the produce increased, it was filled in vials with benzethonium chloride, more commonly known as phemerol as the preservative.

In the beginning, the components of the new combined antigen appeared to be reasonably compatible and the protective antigen stable.

However, in 1960, the Massachusetts Laboratory published a statement suggesting that pertussis antigen was labile in the DPT plus in the poliomyelitis mixture. And subsequently, this statement was confirmed in a special report by Dr. Pittman.

There are many factors in the mixture which may affect the pertussis antigen. One of those factors may be the new chemicals which have been used as preservatives because the poliomyelitis vaccine is not stable in the presence of very small amounts of merthiolate.

We have been interested in the effect on the pertussis antigen of the following chemicals which could be used as preservatives because poliomyelitis vaccine is antigenically stable in them. These chemicals are one in 40,000 dilution of benzethonium chloride, known as phemerol, parabens, mixture of methyl-parahydroxybenzoate, and thirdly, .37 per cent dilution of Beta phenoxy ethanol, commonly known as phenoxetol.

In this study, the stability of the pertussis antigen in plain vaccine and in combination with the other antigens, using these chemicals as the preservatives, is compared with the same products to which no preservative or one in 20,000 dilution of merthiolate was added.

For these studies, the organisms were ~~ground~~ grown for 50 to

52 hours at 36 degrees in a modified Hornibrook medium aerated by bubbling sterile air from the open end of an 8 millimeter glass tube. To kill the organisms, 0.07 per cent formalin was added and the vaccine was detoxified by reincubation at 35 degrees for 72 hours.

The organisms were removed from the culture medium in ~~and~~ a continuous flow centrifuge, and the organisms from about 100 liters of culture were resuspended in 900 cc's of saline containing one in 40,000 dilution of benzethonium chloride. The same lot of concentrated vaccine was diluted to 20 opacity units in ~~and~~ phosphate buffered saline pH 7.2 to prepare the plain vaccine and the DPT.

A different lot of similarly prepared vaccine was diluted in the poliomyelitis vaccine to prepare the quadruple antigen.

These studies are still in progress, but the results to date will be shown in the following tables and the experimental products were stored at 4 degrees and at room temperature. At this higher temperature, any deteriorating effect of chemicals on the antigen would be accentuated.

If we may have the slides, please.

In Slide 1, or Table 1, as some of you may have it, the pertussis vaccine was made up as described and then divided into these fractions -- that containing no preservative, merthiolate, phemerol, phenoxetol, and parabens.

The initial potency of the vaccine was determined on the sample which contained no preservative. These antigenic potencies were done in conventional mouse protection test, using the NIH control vaccine as the standard in each test.

You have here the ~~DP-~~ ED-50 dose of the vaccine reference and over here, this gives us the value in units. And if you work out the 95 per cent ~~competence~~ confidence limits of the ~~task~~ test, you would have this vaccine varying from 4.2 units to 16.8.

After seven months storage at 4 degrees, these products were tested again. And we have here the ED-50 values of the vaccines. And you will note that there is no significant difference in the antigenic value of any of these products except this one which contained phemerol, which is significantly lower than the others and significantly lower than the initial potency.

Now, when you come to the room temperature-stored samples with no preservative, these were tested after eight, twelve, and sixteen weeks. There is no significant difference in the potencies of this.

In fact, there is no significant difference in the potencies of any of these products except the one containing phemerol which after sixteen weeks at room temperature has significant deterioration of antigenicity.

Slide two, please.

Studying the stability of pertussis antigen in DP, the

mixture we were really interested in comparing the stability of DPT containing phenoxetol as compared with the old product containing merthiolate. So we made up three products, one containing no preservative, one merthiolate, one phenoxetol.

The initial potency of the vaccine was determined here, giving us a potency in units with the variation. And then, after seven months storage, we have here the ED-50 values, and there is no significant change in the potency of any of these products at room temperature. The same containing merthiolate, there is no significant change in the potency of it with storage up to twelve months at the room temperature. And the product containing phenoxetol, again, there is no significant change.

But in this product which contains no preservative, there has been a gradual lowering of potency. During the storage period and at the end of the twelve months' storage, it may be calculated that there has been a deterioration approximating 8 per cent per month which is significantly different from zero in this product.

We do not have tests on the plain vaccine to show whether there would be this same deterioration with pertussis antigen not combined with diphtheria and tetanus.

Third slide, please.

The product containing the DPT plus poliomyelitis vaccine, of course, we are interested in the effect of the

preservatives phemerol, phenoxetol and parabens, and we made up the fourth product which contained no added preservative mixture.

The initial potencies of these products were determined, and this one containing phenoxetol gave a somewhat lower result in this test than the other three which were remarkably similar in potency.

After eight months' storage at 4 degrees, there is no significant change in the potency of these preparations. And even the one containing phemerol has shown no deterioration.

In the samples which were stored at room temperature, if you take this product containing no preservative, there has been a considerable variation from test to test. But apparently there has been no significant change in the relative potency of the product even after six months' storage at this temperature.

In the product containing phemerol, after twelve weeks' storage at room temperature, there has been a significant change in the potency and a significant deterioration in the antigenicity of this product.

With phenoxetol, you recall that this potency, initial potency, was low. And during all these tests, this product we seem maybe to have had some testing problems. These results are all low, and we have not been able to establish a significant trend in the variations of the antigenicity of this product.

It may be pointed out, though, that after the six months' storage, this product containing phenoxetol is significantly

lower. The product stored at room temperature here is significantly lower than the similar product stored in the refrigerator. And also that the potency of this is significantly lower than the same material containing no preservative.

It was apparently not significantly different from the product containing parabens. And in parabens, there is no significant change in the potency of the parabens with storage at room temperature for the period of six months. And there is no significant difference in this potency of the room temperature stored material as compared with that stored at 4 degrees.

So that in up to six months' storage at room temperature, there has been no significant trend with the relative potency or the ED-50 at the time of storage established for the lots of DPT plus "P" containing phenoxetol, parabens or no preservative. Therefore, there was no difference in stability.

(Applause.)

DR. WILSON: Thank you, Dr. Corkill.

The next paper is to be delivered by Dr. H. D. Anderson, and the title is, "Stability of the Pertussis Component in DPT-polio, Phemerol ~~Pehmerol~~ preserved."

Dr. Anderson.

DR. ANDERSON: We exploded into this field a few years ago by administrative decision, and there are a lot of

(Slide 1)

TABLE I. ANTIGENIC STABILITY OF PERTUSSIS VACCINE IN SALINE.

	INITIAL POTENCY #504			STORED 4°C 7 MONTHS #524			
	ED50 IN CC	REF.	U/ML 95% LIMITS		ED50 IN CC	REF.	U/ML 95% LIMITS
NIH CONTROL	0.0139		8.0	NIH CONTROL	0.0125		
NO PRESERVATIVE	0.0125	1.04	8.96 4.2 - 16.8	NO PRESEVATION	0.0162	.77	6.2 2.9 - 12.5
				MERTHIOLATE	0.0121	1.03	8.2 3.7 - 15.7
				PHEMEROL ***	0.0402	.3	2.4 1.12 - 4.9
				PHENOXETOL	0.0202	.62	4.96 2.4 - 10.1
				PARABENS	0.0303	.41	3.28 1.5 - 6.2

STORED AT ROOM TEMPERATURE

	#506 8 WEEKS			#510 12 WEEKS			#514 16 WEEKS		
SAMPLE	ED50 IN CC	REF.	U/ML. 95% LIMITS	ED50 IN CC	REF.	U/ML. 95% LIMITS	ED50 IN CC	REF.	U/ML. 95% LIMITS
NIH CONTROL	0.0089		8.0	0.0172			0.0113		
NO PRESERVATIVE	0.0202	.46	3.5 1.8 - 7.5	0.0227	.77	6.16 2.6 - 14.8	0.0202	.56	4.48 1.9 - 8.5
MERTHIOLATE	0.0190	.49	3.76 1.9 - 8.0	0.025	.67	5.5 2.3 - 12.9	0.0154	.74	5.9 3.1 - 13.8
PHEMEROL	0.0216	.42	3.12 .6 - 6.9	0.0167	1.07	8.2 3.7 - 20.8	0.0724 ***	.15	0.776 .55 - 2.6
PHENOXETOL	0.0151	.60	5.0 2.3 - 9.9	0.0345	.5	4.0 1.7 - 9.4	0.0279	.41	3.28 1.5 - 6.6
PARABENS	0.0161	.56	4.3 2.2 - 9.3	0.0524	.45	3.6 1.5 - 8.6	0.0203	.56	4.48 2.0 - 9.0

(Slide 2)

TABLE II. STABILITY OF PERTUSSIS ANTIGEN IN DPT

	INITIAL POTENCY			STORED 4°C 7 MONTHS			
	ED50 IN CC	REF.	U/ML. 95% LIMITS		ED50 IN CC	REF.	U/ML. 95% LIMITS
NIH CONTROL	0.0139			NIH CONTROL	0.0125		
NO PRESERVATIVE	0.011	1.26	10.08 4.6 - 18.9	NO PRESERVATIVE	0.0187	.67	5.36 2.6 - 10.7
				MERTHIOLATE	0.008	1.52	12.16 6.0 - 25.3
				PHENOXETOL	0.0433	.28	2.24 1.1 - 4.5

STORED AT ROOM TEMPERATURE

	12 WEEKS			16 WEEKS			12 MONTHS #548		
	ED50 IN CC	REF.	U/ML. 95% LIMITS	ED50 IN CC	REF.	U/ML. 95% LIMITS	ED50 IN CC	REF.	U/ML. 95% LIMITS
NIH CONTROL	0.0234			0.01516			0.0110		
NO PRESERVATIVE	0.0223	1.04	8.3 4.0 - 18.9	0.0475	.32	2.56 1.2 - 5.28	0.0236	0.47	3.74 1.9 - 7.4
MERTHIOLATE	0.0227	1.03	6.7 3.9 - 18.5	0.025	.61	4.88 2.4 - 10.1	0.0078	1.40	11.20 5.6 - 23.1
PHENOXETOL	0.0279	0.84	8.2 3.2 - 15.0	0.025	.61	4.88 3.1 - 9.8	0.0121	0.91	7.3 3.7 - 14.6

TABLE III. STABILITY OF PERTUSSIS ANTIGEN IN DPT + POLIOMYELITIS VACCINE

	INITIAL POTENCY #512			STORED 4°C 8 MONTHS #530			
	ED50 IN CC	REF.	U/ML. 95% LIMITS		ED50 IN CC	REF.	U/ML. 95% LIMITS
NIH CONTROL	0.01014			NIH CONTROL	0.0169		
NO PRESERVATIVE LOT 490	0.01812	.56	4.48 2.1 - 8.8	NO PRESERVATIVE	0.0196	.86	6.9 3.4 - 14.5
PHEMEROL 489	0.0165	.62	4.96 2.4 - 10.2	PHEMEROL	0.0196	.86	6.9 3.4 - 14.5
PHENOXETOL 487	0.0279	.36	2.96 1.4 - 5.9	PHENOXETOL	0.0197	.86	6.9 3.3 - 14.4
PARABENS 488	0.0167	.61	4.88 2.2 - 9.6	PARABENS	0.0132	1.28	10.2 4.96 - 21.8

S T O R E D A T R O O M T E M P E R A T U R E

	#517 8 WEEKS			#520 12 WEEKS			#532 6 MONTHS		
	ED50 IN CC	REF.	U/ML. 95% LIMITS	ED50 IN CC	REF.	U/ML 95% LIMITS	ED50 IN CC	U/ML. REF.	U/ML. 95% LIMITS
NIH CONTROL	0.01396			0.0125			0.0257		
NO PRESLRVATIVE	0.0127	1.14	** 8.8 3.7 - 23.8	0.0418	.3	2.4 1.04 - 4.7	.0296	.87	6.9 3.5 - 14.1
PHEMEROL	0.0392	.33	2.64 1.12 - 7.12	0.1418	.095	0.72 **.33 - 1.6	-	-	-
PHENOXETOL	0.0606	.23	1.84 .8 - 5.12	0.025	.5	4.0 1.9 - 8.6	.0916	.28	2.24 1.1 - 4.6
PARABENS	0.0568	.25	2.0 .8 - 5.12	0.0303	.41	3.3 1.6 - 6.9	.0384	.67	5.3 2.7 - 10.9

things we couldn't do to start with with background information. We simply had to plow ahead and hope we get someplace.

Our first two experimental lots of quadruple antigen were prepared in the Michigan Department of Health Laboratory in April 1961. We were interested at that time in studying methods of preparation and determination of the stability of the components of the quadruple antigen. These lots were prepared by adsorption of the poliomyelitis vaccine on aluminum phosphate at pH 7.0.

Sufficient supernatant was removed so that addition of the required amount of purified diphtheria and tetanus toxoids and pertussis vaccine restored the volume to the original amount. Control preparations were made at the same time and with the same components that were used for preparing the quadruple antigen.

These control antigens were: 1) DTP, aluminum phosphate adsorbed, 2) pertussis vaccine made up in saline, and 3) pertussis vaccine made up in poliomyelitis vaccine.

The amount of aluminum phosphate in the quadruple and triple antigens varied between 1.42 and 1.65 mg. per ml. The concentration of pertussis vaccine was 12.2 O.U. per single human dose of 1.0 ml. All preparations were preserved with 1:40,000 benzethonium chloride.

Potency tests were made on the quadruple vaccines and controls immediately after preparation and after 6, 12 and 24

months' storage at 5 degrees Centigrade. Only the results of the pertussis tests will be reported here.

May I have the first slide, please?

The results are expressed as arithmetic means and are given in figure 1. These represent two lots in each category and may be summarized as follows:

There was some loss in potency apparently in each of the vaccines, but there was less loss in those vaccines to which aluminum phosphate had been added than in the fluid vaccines.

You will note that this is the DTP-polio. You will note here that we get a higher value for DTP suggesting perhaps some aspect of the polio on the combination pertussis vaccine in saline and pertussis vaccine in polio.

These two things, we think, may have suggested, as Dr. Corkill has reported, that the polio itself contributes something to this loss of potency in the pertussis. These results would be in sharp contrast to our own data on DPT, and we have several lots we have tested for at least five-year periods and have not been able to show any loss of potency in the merthiolate preserved product.

The next experiment consisted of the preparation of five lots of quadruple antigen. These were made with different lots of components or in different combinations. The control preparations were:

1) DTP aluminum phosphate adsorbed.

2) As in the previous experiment, pertussis vaccine diluted in saline, and

3) Pertussis vaccine diluted in aluminum phosphate.

The aluminum phosphate content of the five lots varied between 2.0 and 2.15 mg. per ml. All were preserved with 1:40,000 benzethonium chloride. By the time that we were ready to prepare these lots of quadruple vaccine it was recommended that the total human dose contain 14 pertussis protective units. Because the unitage of the first two lots was so close to this value the amount of pertussis vaccine was increased in the five field trial lots to 16 O.U. per single human dose.

Now, may I have the second slide, please?

The results of the initial potency test, and the 3- and 6-month stability tests are shown in this figure. The values shown are arithmetic means of the five lots or if all five were not done, the mean values of the lots tested. I have the actual number on another piece of paper, but by and large, these are all based on five lots and some of these we may only have one or two.

The results shown are for all practical purposes identical to the results of the previous experiment in that there seems to be some loss of potency during storage, but this loss is small and within the normal variation of the tests.

The loss in the vaccine diluted in saline is not as

great as it was in the first experiment. Future tests at 12, 18 and 24 months may, however, show greater loss in the saline diluted pertussis vaccine than shown by the six month stability data.

It is possible, of course, that losses in the antigens containing aluminum phosphate are greater than the results seem to indicate because of destruction of phemerol and/or the polio vaccine, but these may be masked by the so-called adjuvant effect of the aluminum phosphate. We certainly get a sharp difference here.

Finally, filled vials of the first two lots and a more recent lot of quadruple vaccine had been sent twice by ordinary third-class mail a total distance of 1600 miles. This means a shipment from Lansing to Chicago to Powers, Michigan, and back to Chicago and back to Lansing by ordinary mail, no precautions taken to refrigerate, but just as we would normally mail them.

May I have the last slide, please? (Slide 3)

This would indicate to us that there has been no essential effect of mailing. Here on our first two stability test lots, we started control at 5 degrees for four months and got a value of 21.7.

There were two lots here, stored at 5 degrees for three months, and then sent 1600 miles by mail, 24.6.

Here, the same two were stored for ten months and then

sent 1600 miles by mail. And we got essentially the same values. This was one of our regular production lots where we got a control value of 13.4. And for some reason or other, mailing seems to have had an effect.

(Laughter.)

No explanation.

Then, we took these two original lots, stored one at 5 degrees for 12 months, stored another sample of the same one for ten months, and then incubated them seven days at 35 degrees. And essentially the same data.

As shown by other laboratories, our experiments seem to indicate that methods of preparation and testing which we use, there is some loss of potency after mixing these four antigens. The loss of potency has not been sufficient to preclude its distribution on a statewide scale.

The expiration ~~explanation~~ period of six months, according to our data, is certainly conservative. And according to our experience thus far, it is possible to prepare a DTP-polio combination with a useful life of at least 12 months.

(Applause.)

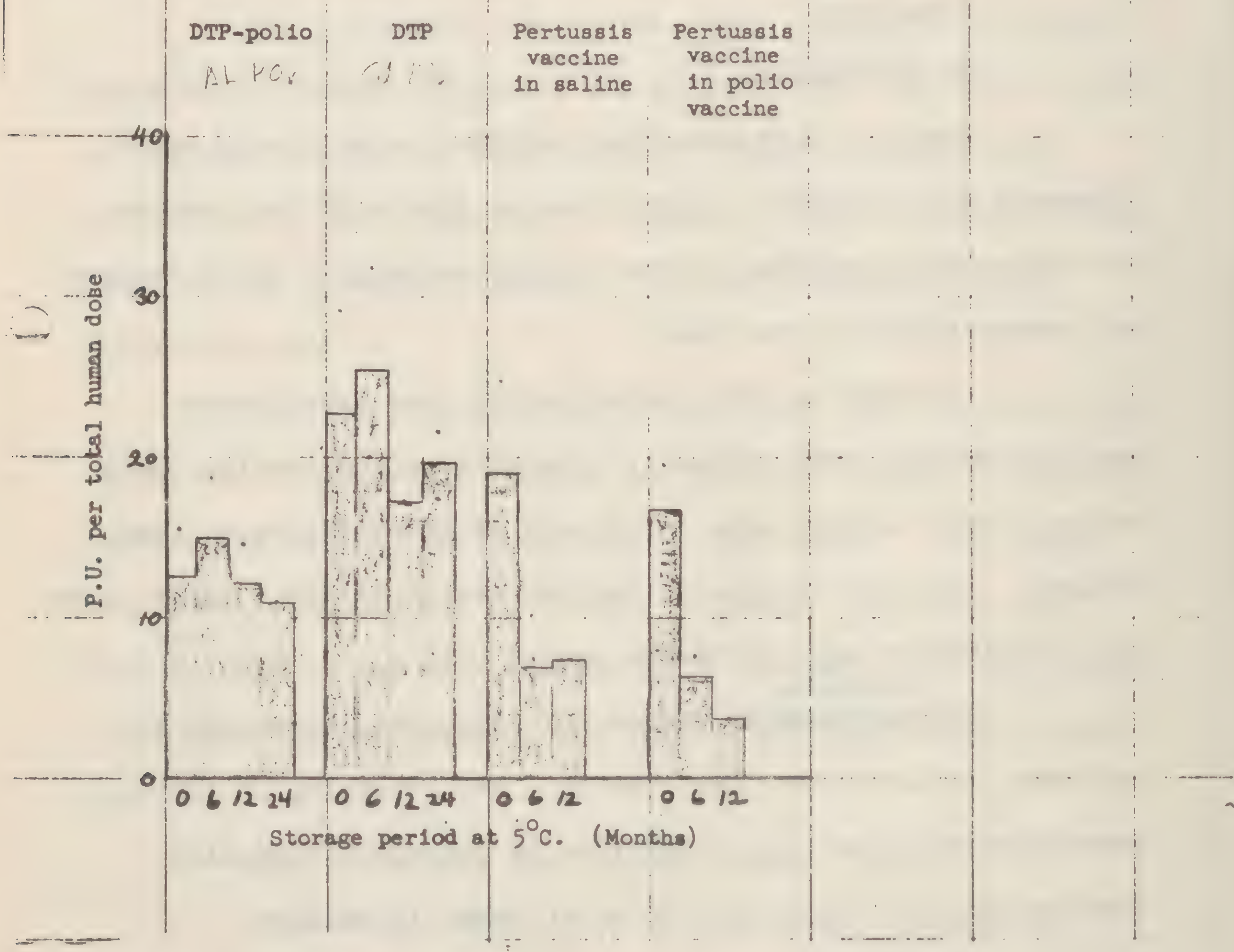
DR. WILSON: Thank you very much, Dr. Anderson.

This strict adherence to time is becoming a fetish, ladies and gentlemen. It is very pleasing.

The third paper by Dr. Birger Olson, Dr. Grace Eldering and Miss Bernice Graham, entitled, "The Stabilization of

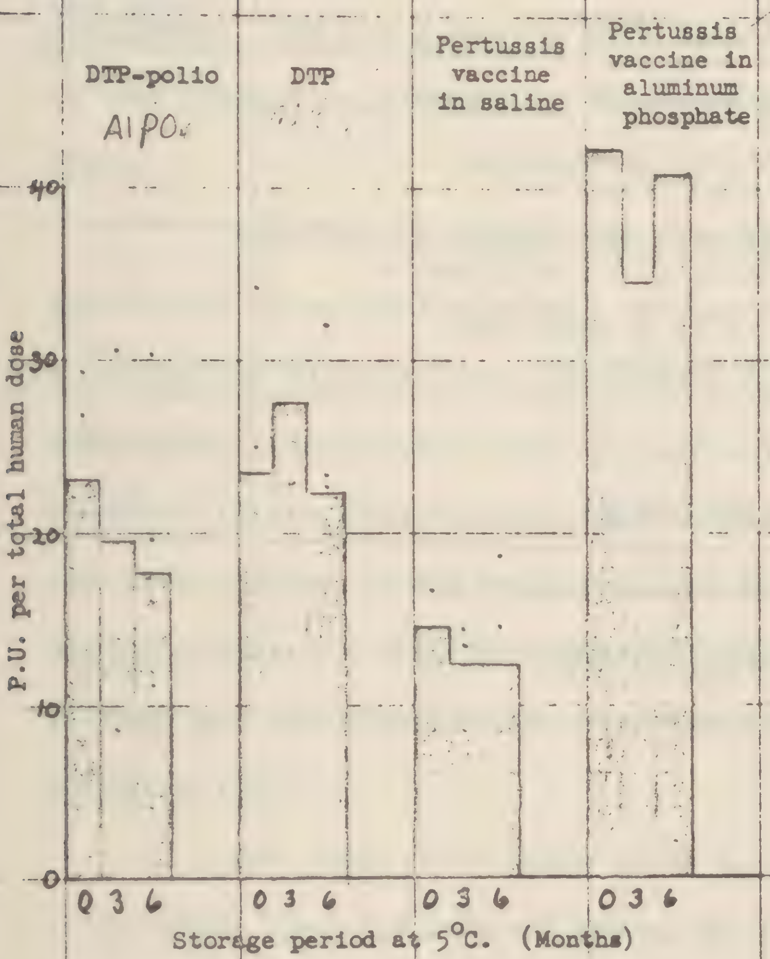
(Slide 1)

Figure 1



(Slide 2)

Figure 2



(Slide 3)

EFFECTS OF STORAGE AND MAILING
ON THE PERTUSSIS POTENCIES OF DTP-POLIO VACCINE

Lot Number	Treatment	P.U. per total human dose (mean)
2001 and 2002	Control. Stored at 5°C for 4 months	21.7
	Stored at 5°C for 3 months then sent 1,600 miles by mail	24.6
2001 and 2002	Control. Stored at 5°C for 12 months	12.1
	Stored at 5°C for 10 months then sent 1,600 miles by mail	15.9
2021	Control.	13.4
	Sent 1,600 miles by mail	39.2
2001 and 2002	Control. Stored at 5°C for 12 months	12.4
	Stored at 5°C for 10 months then incubated 7 days at 35°C	12.5

Pertussis Vaccine in the Presence of Phemerol," will be delivered by Dr. Grace Eldering.

DR. ELDERING: Dr. Wilson, Dr. Pittman, ladies and gentlemen: You are going to gain some more time now. I hope we are going to get overtime, time and one-half, something like that.

Part of the reason for this is that Dr. Corkill has given most of my introduction. He has already stated that it is a recognized finding that the potency of the pertussis component deteriorates in certain lots of quadruple antigen in which phemerol is the preservative, and he has called to your attention the 1960 warning of the Massachusetts Department of Public Health concerning this fact and the comprehensive report of Dr. Pittman and the further report of Edsall, McComb, Wetterlow and Ipsen in 1962.

Now, what do we know about benzethonium chloride? It is known from the work of Kivela in 1948 that when quarternary ammonium compounds function by attachment to the negatively charged sites on the bacterial cell surface, it may be that in this adsorption a denaturation of the cell wall occurs.

Mueller and Seeley (1951) reported that the germicidal action of a quarternary ammonium compound such as Phemerol is much decreased in the presence of metallic ions. The degree of interference with the germicidal action is proportional to the valence of the metallic ion. The interfering activity of mono-

di, and tri-valent ions is approximately in the ratio of 1 per mono-valent to 100 per di-valent, and to 10,000 per tri-valent ion.

Mueller and Seeley state that the results of their studies support the theory that any metallic cation can interfere with the adsorption of quarternary ammonium compounds by competing for the negative sites on the bacterial cell surface. The same authors state that the higher the valence of the ion, the more strongly it is attached and held to the surface of the organism. The negative charge on the surface of the cell is reduced, thus lowering the attracting power of the cell for the quarternary ammonium compound.

These results suggested to the senior author, Dr. Olson, that it might be possible to saturate the negative sites on the pertussis cell prior to the addition of the preservative, Phemerol, thus preventing its uptake by the cell and thereby stabilizing the antigenic potency of the vaccine.

The present experiments were designed to investigate the effect of the addition of cations to pertussis vaccine before Phemerol.

The next part of this, I hope, means more to you than me.

Phemerol was detected and identified by its characteristic ultraviolet absorption spectrum. A Cary Model 14 Spectrophotometer was used for all ultraviolet absorption determinations.

Absorption maxima were found at 2745 Å, 2683 Å and 2630 Å. Shoulders on the curve appeared at 2820 Å and 2560 Å. In order to measure the Phemerol concentrations quantitatively, a family of curves was obtained at concentrations between 25 and 200 ppm.

The eosin yellowish quarternary ammonium dye complex method of Furlong and Elliker (1953) and Miller and Elliker (1959) was used to determine the concentration of unadsorbed Phemerol. This method was modified from a titrametric to a colorimetric method.

When appreciable amounts of cation were present in the solution, the extract of the dye complex was washed with water to remove excess cations and so allow for color development. Color standards of Phemerol-dye complex containing 5, 10, 15, 20 and 25 mcg Phemerol were prepared and used to determine concentration.

The ultraviolet absorption curves for pertussis vaccine supernatant were determined at pertussis cell concentrations from 15 billion to 480 billion cells per ml. When a small amount of ultraviolet absorbing material was present, such as in the more dilute vaccines, Phemerol was identified by the ultraviolet absorption curve and measured by the increase in absorbance at 2683 Å.

In vaccines which had appreciable ultraviolet absorption, as in the higher cell concentrations, the Phemerol was measured by the dye complex method.

In order to determine unadsorbed Phemerol the pertussis vaccine was filtered through a Millipore filter and the determination carried out on the filtrate. A membrane filter was used because ultrafine fritted glass filters of Seitz filters adsorbed all of the Phemerol from the solution.

The potency of the pertussis vaccine was determined by the standard mouse-protection test. White Swiss mice from the Michigan Department of Health colony were used in these tests.

The pertussis vaccine used in these experiments was a lot prepared by Dr. Anderson from one strain of pertussis, 10536, and all experiments were carried out on pertussis vaccine alone, not on a combined product containing polio or any other antigen. We used culture 10536 because this culture is well known to all of us and has been extensively studied in our own and other laboratories, and its characteristics, particularly with respect to mouse protection, are well known.

The antigen was grown on Bordet-~~gengou~~ Gengou medium according to our usual method preparing pertussis vaccine.

And now, may we have the first slide?

The ability of cations to interfere with the adsorption of Phemerol on the pertussis cell is shown here. Each cation was added to a portion of vaccine at 15 billion cells per ml and thoroughly mixed prior to the addition of Phemerol.

Here we have calcium chloride in two different concentrations, 0.001 M and 0.002 M, lysine and aluminum

potassium sulphate. This is in a 0.0002 M. Where we had only Phemerol, no unadsorbed Phemerol could be detected in the supernatant. It was all adsorbed on the cells.

The calcium chloride in this concentration prevented the adsorption of about half of the Phemerol. Here it was all present in the filtrate. Here (indicating) about a third. And the aluminum potassium sulphate trivalent metallic ion prevented all of the adsorbed DL-lysine.

It is interesting to note that the amino acid lysine competitively interferred with the adsorption when present in the concentration shown.

Although not listed, magnesium and choline were also able to prevent the adsorption of the preservative.

Based on the results shown here, a series of vaccines was prepared which contained cations added prior to the addition of the preservative. These vaccines were stored at zero to 4 degrees Centigrade, and their potencies were determined at intervals throughout a 12-month period, and the results in protective units per ml of a 10 billion or 10 opacity unit per ml vaccine are shown in the next slide. (Slide 2)

There were many more tests done than are shown here, but we wanted to make it reasonable.

The number refers to the vaccine containing "none", only Phemerol, and here we had an initial value obtained by averaging these tests and a few others of close to 5 protective

units per ml of a 10 billion per ml vaccine at the beginning of the experiments.

The Phemerolized vaccine decreased in potency at 30 weeks, and here it had gone clear down, and here (indicating) it was .9. That is, it was practically nonpotent.

The other vaccines did not decrease in potency during the year's storage except for the choline, and that perhaps is an inconsistency of the testing.

Now, in order to accelerate the tests, we started one set of vaccines at 37 degrees. This was something like Dr. Corkill's accelerated test except we used a higher temperature. We used incubator temperature.

And I guess we should have the next slide. (Slide 3)

We have the same initial tests here. These are averaged. It comes, I think, to 4.85 or close to 5 protective units per ml. At 10 weeks, the merthiolate vaccine had gone down. The merthiolate vaccine showed no protection at 42 weeks.

The Phemerol only was practically gone at 10 weeks and all gone at 16 weeks.

The calcium chloride still had, and the aluminum phosphate sulphate still had about 80 per cent of their protection at the end of 42 weeks, and we have tested the aluminum potassium sulphate vaccine at 14 months and in the highest dose tested, which is the 1.5 billion, 16 of 32 mice survived which really is almost unbelievable.

It is realized that these experiments refer to only one lot of pertussis vaccine. However, the data show that under the conditions used, cations prevented the adsorption of Phemerol onto the pertussis cells and stabilized the antigen as measured by the mouse-protection test.

It should also be noted that the accelerated potency test -- that is, storage at 37 degrees -- was very useful in these experiments.

In summary, data have been presented showing that pertussis vaccine treated with Phemerol is inactivated on storage. Phemerolized vaccine stored at 37 degrees Centigrade was inactivated in 16 weeks -- actually in 10. Phemerolized vaccine stored at zero to 4 degrees Centigrade lost approximately 80 per cent of its protective antigens in one year.

It was shown by chemical and physical means that aluminum, calcium, magnesium, choline, and lysine when stirred with pertussis vaccine prior to the addition of Phemerol prevented the uptake of Phemerol by the pertussis cells.

Pertussis vaccine to which was added either 0.004 M calcium plus plus or 0.0004 M aluminum plus plus plus before Phemerol retained 70 per cent of the initial protective antigen during 42 weeks storage at 37 degrees Centigrade. These same vaccines showed no loss of protection when stored for one year in the icebox.

(Applause.)

(Slide 1)

Effect of Cations on the Adsorption of Phemerol by
B. pertussis cells
Phemerol 25 ppm added after supplement

Supplement added	Concentration of Supplement	Unadsorbed Phemerol ppm	Unadsorbed Phemerol Percent
None	0	0	0
Calcium Chloride	0.001M	12	48
Calcium Chloride	0.002M	25	100
DL Lysine	0.007M	8	32
Aluminum potassium sulfate	0.0002M	25	100

(Slide 2)

Results of mouse protection tests on vaccines stored at 0-4C

Supplement to Phemerolized vaccine	Concentration of Supplement Added	Protective units/ml of a 10 Bil/ml vaccine			
		0 Weeks	30 Weeks	39 Weeks	52 Weeks
None		4.7	1.9	2.0	0.9
Calcium Chloride	0.004 M	3.6	2.3	5.6	5.2
Aluminum Potassium Sulfate	0.0004 M	4.3	6.9	7.5	5.4
Magnesium Sulfate $7H_2O$	0.0008 M	7.1	6.9	3.8	5.0
Choline	0.0008 M		4.7	5.8	2.7
Calcium Chloride DL Lysine	0.002 M 0.007 M		2.1	3.9	4.9

(Slide 3)

EFFECT OF CATIONS ON THE STABILITY OF PERTUSSIS VACCINE PRESERVED WITH 25 PPM PHEMEROL AND STORED AT 37 C

Vaccine	Protective Units/ml of Vaccine containing 10 Billion organisms/ml				
	0 Weeks	5 Weeks	10 Weeks	16 Weeks	42 Weeks
Merthiolate (No phemerol)	4.6	2.1	2.1		No protection
Phemerol only	4.7	2.8	.8	No protection	No protection
Calcium chloride + Phemerol	3.6	3.6	3.0	3.6	3.3
Aluminum potassium sulfate + Phemerol	4.3	8.5	3.8		3.4

DR. WILSON: Thank you very much, Dr. Eldering.

The next paper is again by Dr. Anderson entitled, "Agglutinin Response in Infants to the Pertussis Component of DTP-polio, Phemerol Preserved."

DR. ANDERSON: During the past several years, we have performed potency tests on a variety of multiple antigens containing pertussis vaccine. We have also participated in field trials for the evaluation of quadruple vaccines containing diphtheria, tetanus, pertussis and poliomyelitis antigens Phemerol preserved. In the course of these studies we have measured the agglutinin response in infants to products with poor pertussis potency as measured by the mouse-protective test, as well as to products with satisfactory pertussis potency. The results of these studies form the basis for this report.

(Slide 1)

A field trial in Saginaw, Michigan, started in June, 1960, was designed to compare nine dosage schedules for the immunization of infants with a quadruple antigen. This trial was designed by the Multiple Antigen Committee of APHA and was conducted by Dr. V. K. Volk, Dr. Gordon Brown, Dr. Pearl Kendrick, Dr. R. Y. Gottles and H. D. Anderson. Shortly after the study was started, reports were received from the Massachusetts laboratories that several lots of commercial DTP-polio lost pertussis potency very rapidly.

We continued the trials, fully expecting that the pertussis response might be poor. We tested the lot of DTP-polio

used in the trial at intervals of 4 and 5 months from the date of release by the manufacturer.

The Massachusetts laboratories also tested this lot on two occasions. The combined data indicated that this lot (A) may have lost potency from 28 protective units per total human dose to less than 5 protective units within 5 months of the release date.

That (indicating) was this lot. This was the Massachusetts first test.

Here was their second test,

This was our first test and our final test on the lot. The manufacturer kindly supplied a second lot (B) which had been released in October, 1960, with a potency of 16.5 P.U/THD. This lot was used beginning in January, 1961, for all new admissions to the study.

In other words, if you note here (indicating) Lot A was used for primary immunization of certain groups of children during this period and for boosters here.

This lot (B) was used here when we found that this potency was down. And those primaries extended to here with booster out to this point.

In the meantime, the problem of vacuolating virus (SV-40) had been recognized in the polio components and the requirements for pertussis potency in DTP-polio vaccines were also changed to a minimum of 14 P.U/THD. The manufacturer

suspended production of further lots, so we decided to continue the study using the two lots past their expiration dates and expecting poor pertussis results. However, the supply of lots A and B was insufficient to complete the immunizations of the desired number of infants.

The Michigan Department of Health Laboratories were actively engaged in the development of a DTP-polio vaccine Phemerol preserved and had prepared two lots to study the stability of each component. These were described in our previous report. To extend the Saginaw trials, we furnished sufficient quantitites of our "stability test lots" to complete the immunizations of all additional infants.

All infants received their primary injections at one-month intervals. These lots were prepared before the change was made in minimum requirements for the pertussis component came out. That is, our stability lots.

Early in 1962 we prepared five lots of DTP-polio for field trials which were evaluated in infants by Dr. C. D. Barrett, Jr., in Detroit, Michigan, and Dr. Barrett prepared some of the reaction data on these lots yesterday. These lots had an initial potency of 23 PU/THD and remained at approximately the same level when tested six to eight months later. That's this group. These are the tests on our stability test lots which show that 24 months' stability with the loss of essentially one unit of potency which indicates to us pretty good stability.

This material was given in primaries here and in boosters here (indicating). We do not have enough data here. We have had too many other testing problems to do a 12-month, but we do have one test at about 12 months, and we have one test that came off last week on samples that had been returned outdated from the field. And it was on one of the field trial lots. And this came out 15 protective units.

Now, this is why it is not put on here. We simply have the one, but it does represent something that has been in the field and has come back outdated and was tested at approximately 18 months with a potency of 15 protective units.

May I have the next slide, please? (Slide 2)

These data, based on the Saginaw trial reflect the pertussis agglutinin responses in infants to lots of DTP-polio which had decreased to less than 5 protective units by the time they were injected into most of the children. A preprimary titer of 5 was arbitrarily assigned to any value less than one to 10 or a value of ten just so we can calculate geometric means. Neither the age of the child nor the dosage scheduled seemed to have had much effect upon the response, although if you take a child with a 3-4-5 and compare it with a 3-5-7, you see a little value change, or 4-5-6 with a 4-6-8 show a little change.

This is the subject of another report that will be given in Kansas City in November, and it is much more striking for the other components whereas the potency is present.

But age at determination of the primary seems to be one of the most important points to be brought out in this study of dosage schedules. But generally, the agglutinin response was low. But a detectable response did occur both to the primary and to the booster.

I mean, even though the potency is largely gone, you do see stimulation. And it is interesting that in this three-year period covered by the study, we have had one case of pertussis in this group of infants. Surveillance, of course, would be continued, and we can't say much more about it than that.

May I have the next slide, please? (Slide 3)

These data refer to a similar but much smaller group of infants in the Saginaw trials. These infants received their primary injections at one month intervals. And as in the first group, the boosters were given one year after the first primary injection and the volume of the booster was .4 of a single immunizing dose.

In other words, our regular dose is 1 ml, and these youngsters were given .4 ml as a booster.

These infants received a fully potent pertussis component and good responses in pertussis agglutinins occurred as a result of the primary series.

At the end of one year, the prebooster titers were somewhat higher than the preprimary titers, of course. But the

post-boosters appeared to be somewhat lower in three of the groupings than we got post-primary.

In the Saginaw trials, using the MDH vaccine, the majority of children received a primary series beginning at three and four months of age, as you will note. But you will recall from the previous slide that these showed among the lowest response titers.

May I have the next slide, please? (Slide 4)

This reflects the field trials on our so-called field trial lots. Dr. Dale Barrett did the field work for us, and we did, of course, all the laboratory work on the serological work.

Most of our children were between five and eleven months of age because we were deliberately selecting children where the probabilities of obtaining no polio antibodies were best.

In other words, by waiting this long, we were trying to avoid maternal antibody which might affect the polio response.

The lots used in this trial had an initial potency of 23 protective units per total human dose and remained approximately at this level through the period of the trials.

The responses following the primary series of injections were considerably higher than in the Saginaw trials. Here, you will notice post-primary titers of 928 for the younger

group of children, 1,000 for those that are started at six months of age, 780 and 799 for the older children.

There was a drop during the eleven months between the primaries and the booster, but one interesting thing here is that these values are roughly half the post-primary titer. There is no explanation for this except that these children received the full immunizing dose and this may have some interesting points to take off on discussion.

In summary, then, using multiple vaccines containing diphtheria, tetanus, pertussis and polio antigens, the response in infants to the pertussis component was evaluated.

When the pertussis potency had fallen below 5 PU/THD early in the trials the pertussis agglutinin titers were low and showed little difference, based upon the age of the child or dosage schedule.

When a potent pertussis component was used in children three to four months of age at the start of the primary series, and a booster of 0.4 of a single dose was given one year later, the response was much better.

When a vaccine with a high pertussis potency was used in children five to eleven months of age at the start of the primary, the pertussis agglutinin titers were the highest of the three groups. The booster dose was a full single dose but the titers observed two weeks following the booster were lower than the post-primary titers.

Thank you.

(Applause.)

DR. WILSON: Thank you, Dr. Anderson.

Now, due to the strict adherence to time, we have gained a little, and I think at the moment we will take a break for coffee. And if you would please return not later than twenty minutes from now, which would be 20 minutes to three.

(Whereupon, a recess was taken.)

DR. WILSON: Ladies and gentlemen, if we could bring the meeting to order.

The next paper is to be presented by Mrs. Roberta A. Gardner, and it is entitled, "Rapid Assays for Detecting Loss of Potency."

Mrs. Gardner.

MRS. GARDNER: I think maybe a more appropriate title would be, "Rapid Assay, Question Mark."

Because the current potency test for pertussis vaccine requires one month to complete, and is expensive to perform, it would be desirable to have a more rapid assay, especially for research purposes. We are not presenting you with the solution to the problem today, but rather we would like to stimulate you to apply your special knowledge to the problem.

Two approaches might be considered:

1) Ideally a test would be developed which

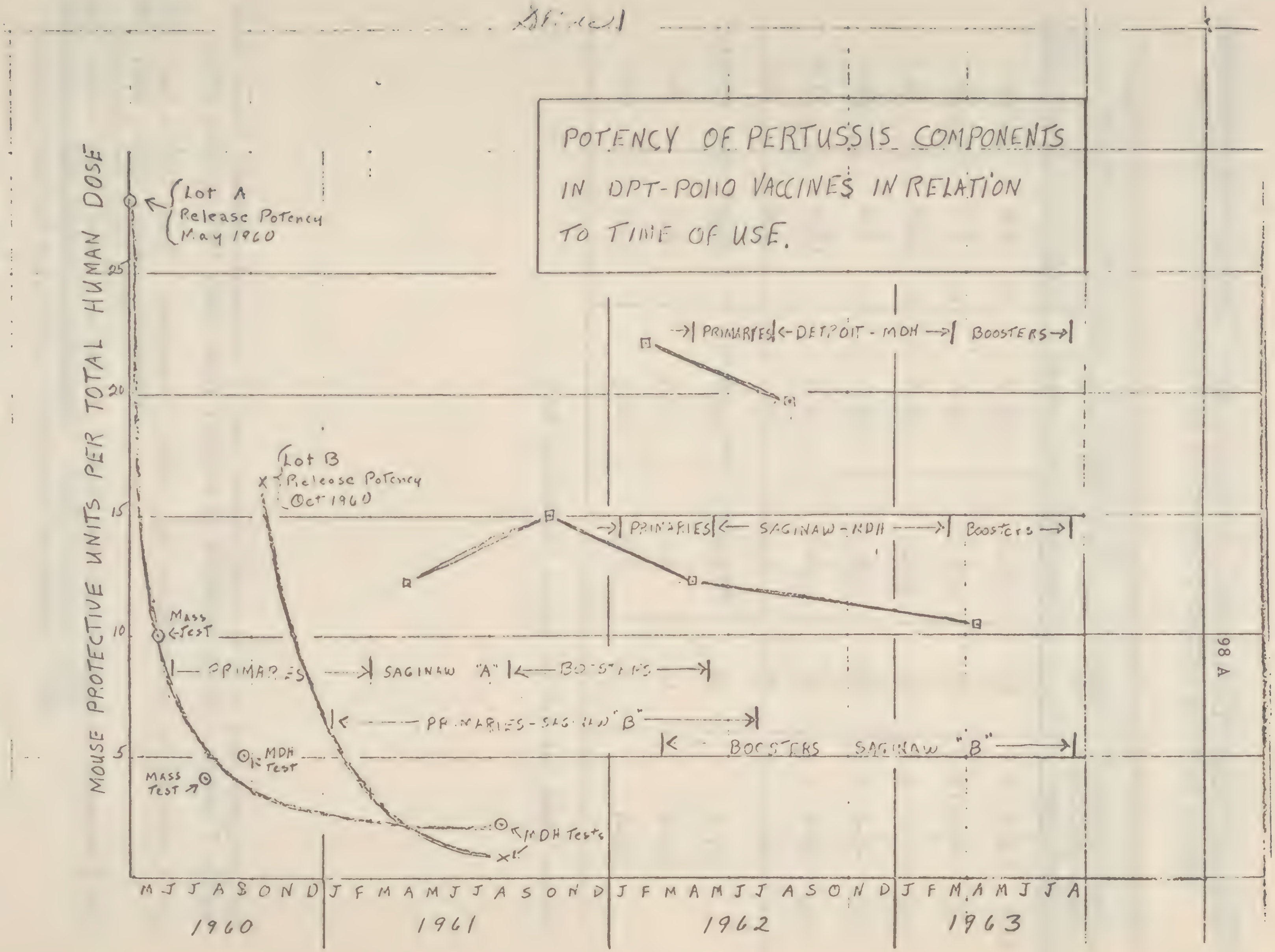

POTENCY OF PERTUSSIS COMPONENTS
IN DPT-POLIO VACCINES IN RELATION
TO TIME OF USE.
MOUSE PROTECTIVE UNITS PER TOTAL HUMAN DOSE
25
20
15
10
5
Lot A
Release Potency
May 1960
Lot B
Release Potency
Oct 1960
Mass Test
Mass Test
MDH Test
MDH Tests
PRIMARIES
SAGINAW "A"
BOOSTERS
PRIMARIES - SAGINAW "B"
BOOSTERS SAGINAW "B"
PRIMARIES
SAGINAW - MDH
Boosters
PRIMARIES
DETROIT - MDH
BOOSTERS
M J J A S O N D J F M A M J J A S O N D J F M A M J J A S O N D J F M A M J J A
1960
1961
1962
1963

Table 2

PERTUSSIS AGGLUTININ RESPONSES IN INFANTS GIVEN 9 DIFFERENT DOSAGE SCHEDULES USING A MULTIPLE ANTIGEN (COMMERCIAL) CONTAINING DTP-POLIO ANTIGENS

Dosage Schedule Ages of Child in Months	Number in Group	Agglutinin Titers (reciprocal of dilution) Expressed as Geometric Mean Titers			
		Preprimary	Postprimary	Prebooster	Postbooster
3-4-5	44	6.0	19.7	6.8	23.4
3-4-5-7	28	5.7	25.6	7.7	36.7
3-5-7	31	7.3	24.5	10.0	33.3
4-5-6	59	6.3	16.4	7.4	24.0
4-6-8	50	6.7	19.5	18.4	33.0
5-6-7	35	6.5	29.7	10.0	37.6
5-7-9	28	6.4	29.7	10.7	38.7
6-7-8	15	6.0	30.3	12.1	50.3
7-8-9	6	7.9	28.3	12.6	25.2

Booster dose was 0.4 of single immunizing dose

The antigens used in this study showed loss of pertussis potency (mouse test) within 7 months. Study extended 35 months, from June, 1960 to May, 1963.

Lot A released by manufacturer May, 1960.	Primaries:	June, 1960 - May, 1961
	Boosters:	Aug., 1961 - May, 1962
Lot B released by manufacturer Oct. 1960.	Primaries:	Jan., 1961 - Aug. 1962
	Boosters:	March 1962 - Aug. 1963

Slide 3

PERTUSSIS AGGLUTININ RESPONSES IN INFANTS GIVEN 5 DIFFERENT DOSAGE SCHEDULES USING A MULTIPLE ANTIGEN (MDH) CONTAINING DTP-POLIO ANTIGENS

Dosage Schedule Ages of Child in Months	Number in Group	Agglutinin Titers (reciprocal of dilution) Expressed as Geometric Mean Titers			
		Preprimary	Postprimary	Prebooster	Postbooster
3-4-5	13	5.0	93.9	9.0	70.2
4-5-6	20	5.0	114	8.6	92
5-6-7	5	5.0	160	13.2	183
6-7-8	2	5.0	320	25.2	450
7-8-9	7	5.0	353	16.4	160

Booster dose was 0.4 of single immunizing dose

Manufactured April, 1961.

Primaries started January, 1962. Completed May, 1962.
Boosters started May, 1963. Completed September, 1963.

Slide 4

PERTUSSIS AGGLUTININ RESPONSES IN INFANTS GIVEN 3-DOSE PRIMARY AT 1-MONTH INTERVALS PLUS A BOOSTER DOSE USING DTP-POLIO

Primary Started: Age in Months	Number of Children	Agglutinin Titers (reciprocal of dilution) Expressed as Geometric Mean Titers			
		Preprimary	Postprimary	Prebooster	Postbooster
5	54	20.5	928	41.0	447
6	43	23.1	1005	49.3	442
7	21	20.0	780	31.7	299
8-11	25	17.4	799	32.1	338

specifically measures the protective antigen.

2) A test might be employed which may or may not actually measure the protective antigen, but which correlates well with potency as measured in mice and therefore is useful, perhaps only under certain specified conditions or perhaps only as a screening test. Types of tests which might be considered would be other animal assays, *in vitro* chemical assays, and *in vitro* immunological assays.

First we might consider the possibility of other animal assays. Although authors disagree as to whether the histamine-sensitizing antigen and the protective antigen are an entity, in ordinary pertussis vaccines there is frequently a close relationship between the histamine-sensitizing ability of the vaccine and its ability to protect mice against intracerebral challenge with virulent pertussis organisms. Since a histamine-sensitization test can be completed in five days, as compared to the 28 days required for a potency test, thus saving time and reducing the space required for housing mice, there may be situations in which it would be useful.

Could we have the first slide, please?

You can see here that vaccines which did protect mice also were fairly good as sensitizing mice to histamine whereas vaccines which were lacking in potency were very poor or completely lacking in histamine-sensitizing ability.

Second slide, please.

As you know, heating a pertussis vaccine at 70 degrees Centigrade will destroy potency. As you can see, this treatment also destroyed most of the histamine-sensitizing capacity of the vaccine. Boiling it for 30 minutes completely destroyed all histamine-sensitizing capacity.

Slide three, please.

The effect of preservatives upon the stability of pertussis vaccine has been studied. Here, too, there appears to be a relationship between the potency test and the histamine-sensitization test, although not a perfect relationship. The histamine test could distinguish a vaccine with 8.0 protective units per ml from 4 vaccines with values of 2.5 to 4.7 units per ml.

It did not, however, distinguish between the 4 vaccines. These 4 vaccines were placed at 35 degrees Centigrade for 48 hours and then held at 4 degrees Centigrade for three to four weeks before being tested.

As you can see with the merthiolate preserved vaccine, there was no loss in potency. There also appeared to be no loss of histamine-sensitizing factor.

With the vaccine with no preservative, no change in histamine-sensitizing capacity could be detected, although there probably was some loss in potency.

This value, however, still falls within this range of 2.5 to 4.7 units, so we might not expect to be able to detect

any difference.

With the benzethonium chloride preserved vaccine, there was some loss in potency, also some loss of histamine-sensitizing capacity.

There was a slight decrease in number of deaths. In addition, the time required for the mice to begin dying increased.

At this dose, for example, it took something like 12 minutes here for death to occur as against 25 minutes here (indicating).

With the vaccine preserved with methyl- and propyl-parahydroxybenzoate here (indicating), it took 9 minutes for death to occur, here 20 minutes.

In addition, there was a decrease in number of deaths. There was also a decrease in potency.

You will note after storage at ~~3~~ 35 degrees, both of these two vaccines had potency values of two or less, and the histamine test apparently could distinguish them from the vaccines with 2.5 or more units of potency.

It would appear that testing for histamine-sensitizing factor may be useful at least in some situations as a relatively rapid test for determining whether a pertussis vaccine is potent.

Secondly, we might consider the general field of the possibility of chemical assays. If eventually we find out what

the chemical makeup of the protective antigen is and if it should happen that changes which destroy vaccine potency also produce measurable changes in a chemical component making up the protective antigen, then it might be possible to devise some chemical test which would reflect vaccine potency.

This approach, of course, is complicated by the presence in most vaccines of a great deal of extraneous material from the bacterial cell or the media which has nothing to do with potency, and also by the possibility that the changes required to destroy potency may be very small and not measurable by any test.

Since it is probable that protein is associated with the protective antigen of pertussis, the possibility was investigated that changes in the protective antigen would be reflected in changes in the biuret reaction. There did appear to be a relationship between the biuret reaction of a vaccine and its potency.

However, the relationship was between the biuret reaction and the original potency of the vaccine, not between the biuret reaction and losses of potency. There was, unfortunately, not a correlation between the biuret reaction and the present potency of the vaccine if the vaccine had declined in potency upon storage.

In studying the relationship between the potency of plain pertussis vaccines and the biuret reaction it was necessary to also consider the opacity of the vaccine and the manufacturer

of the vaccine. The products of different manufacturers seemed to fall into one of two groups. Products of seven manufacturers behaved similarly. Vaccines produced by four others behaved in a slightly different manner. Within each of these two groups the opacity of the vaccine was also considered.

Slide four, please. (Slides 4a and 4b)

If vaccines of similar opacity were tested, there was a relationship between potency and the biuret reaction. (Slide 4a)
Over here (indicating) are vaccines of 12 to 20 opacity units per ml produced by a group of seven manufacturers.

In this section here are vaccines produced by the same manufacturer, but of higher opacity. (Slide 4b)

In here are vaccines 15 to 30 opacity units per ml produced by a second group of four manufacturers.

Now, in looking at these graphs, we need to remember that the variability of the potency test is quite large. When potency is plotted versus optical density at 555, it is found that a given optical density reading corresponds to about a twofold range of potency values. A twofold range is about as good as can be expected from the limitations of the mouse test unless a large number of tests are performed for each value.

On the graph, lines were drawn to enclose a twofold range of potency values. For example, here (indicating), at a reading of about 0.08, you range from 3 to 6 units whereas up here you have a range of about 7 to 14 units with vaccines

produced by the same manufacturer, but of a higher opacity.

Over here, a given optical density reading corresponds to a potency of about twice as high.

It is also rather interesting to note that these vaccines of low opacity produced by this group of manufacturers behave quite similarly to those of higher opacity produced by a different group. So it does appear that when the vaccines are grouped by manufacturer and opacity, it might be possible to determine the original potency of the vaccine within a twofold range.

However, it is not much good for necessarily determining the present potency of the vaccine.

A number of these lots of vaccine have been retested anywhere from one to seven years after the original potency tests. For example, this one which was tested originally about 1958, in 1963 has a potency value of 1.4 which would fall down in this area (indicating).

There are a number of others whose potency now is completely ~~slightly~~ lacking or very low, and there is no relationship between that present potency and what you see on this reaction. If you use precipitated or adsorbed ~~adsorption~~ pertussis vaccines rather than plain pertussis vaccines, you see a similar pattern except that since the adjuvant increases the immunizing capacity of the vaccine for a given optical density reading gives a higher potency value.

May I have slide five, please?

If we ignore all attempts at grouping the vaccines by opacity or manufacturer and simply plot the optical density of all plain vaccines versus potency, in general an increase in optical density still corresponds to an increase in potency.

However, for a given optical density, there is about a four-fold range in potency.

Slide six, please.

Excessive heat destroys the potency of pertussis vaccine, and it was thought that perhaps heating the vaccine would do enough damage to be detectable in biuret reaction. However, instead of observing any decrease in optical density with decrease in potency, a very small but consistent increase in optical density was found.

Since the protective antigen of pertussis is closely associated with the cell wall, perhaps sometime there might be a possibility of correlating some cell wall component with potency assay.

Since sialic acid has been reported to be a constituent of bacterial cell walls, the relationship of sialic acid content to pertussis vaccine potency was investigated. The thiobarbituric acid method of Warren was employed.

Slide seven, please.

Unfortunately, however, there was not any correlation between the potency of the vaccines and their sialic acid

content.

Commercial vaccines of known potency have also been tested and no correlation was found.

The third general area in which some day we might hope to have a more rapid immunization would be *in vitro* immunological assays.

I have not done any experimental work in this area, so I can speak only in generalities.

Of course, a major step in employing *in vitro* assays for the detection of protective antigens is the preparation of purified materials so that it is known that protective antigen and not some other antigen of the bacterial cell is being measured.

In recent years, many workers have been attempting to purify protective antigen. Preparation of purified protective antigen, in turn, makes possible the preparation of specific antisera.

There are a number of *in vitro* systems in which purified protective antigen and antibody might possibly react. For example, they may form a characteristic precipitation line in an Ouchterlony plate which correlates the immunizing activity, or possibly the antigen could be labeled with some radioactive isotope and employed in a radioisotope precipitation test in which labeled antigen, antibody, and antigammaglobulin form a radioactive precipitate.

Whatever system might be employed in measuring the antigen-antibody reaction, it might be necessary when measuring the protective antigen of an ordinary vaccine, rather than purified protective antigen, to employ some sort of inhibition test in which the vaccine is combined with specific antisera. The antisera is then combined with the purified antigen,and the protective power of the vaccine is measured by how much it has inhibited the reaction of the purified antigen with the antibody.

There is one serious problem in any of these *in vitro* systems. The ability of protective antigen to combine *in vitro* with specific antibody does not necessarily mean that the protective antigen has the ability to produce antibody *in vivo*

It is possible to have a somewhat degraded antigen which still retains the ability to combine with antibody, even though it has lost the ability to produce protective antibody.

The problem of rapid assays for pertussis potency certainly has not been solved, but perhaps some of you may have some helpful ideas.

(Applause.)

DR. WILSON: Thank you very much, Mrs. Gardner.

Ladies and gentlemen, firstly, I would like to thank all of those who presented such interesting papers this afternoon and certainly provide room for ample discussion. I think only a group such as this can realize how much work and effort

(Slide 1)

Sensitizing Vaccine 7.5 op.u.	Histamine Diphosphate mg/kg	Deaths/No.		Potency Units/ml
		Test 1	Test 2	
Lot 2	25		1/10	2.7 u/ml.
	50		6/9	(8 tests)
	100	6/10	7/9	
	200	5/10		
	400	3/10		
5	25		1/10	1.9 u/ml.
	50		5/10	(5 tests)
	100		6/10	
3	100		0/10	Nil
	200		0/10	(4 tests)
	400		0/10	
4	100		4/10	Nil (5 tests)
	200	0/10	0/10	1.0 u/ml. (1 test)
	400	0/10	0/10	
	800	0/10		

(Slide 2)

SENSITIZING CAPACITY OF PERTUSSIS VACCINE BEFORE AND AFTER HEAT TREATMENT

Histamine Diphosphate mg/kg	Before Treatment	70 °C 30 Min.	100°C 30 Min.
25	1/10		
50	6/9		
100	7/9	3/10	0/10
200		2/10	0/10
400		1/10	0/10

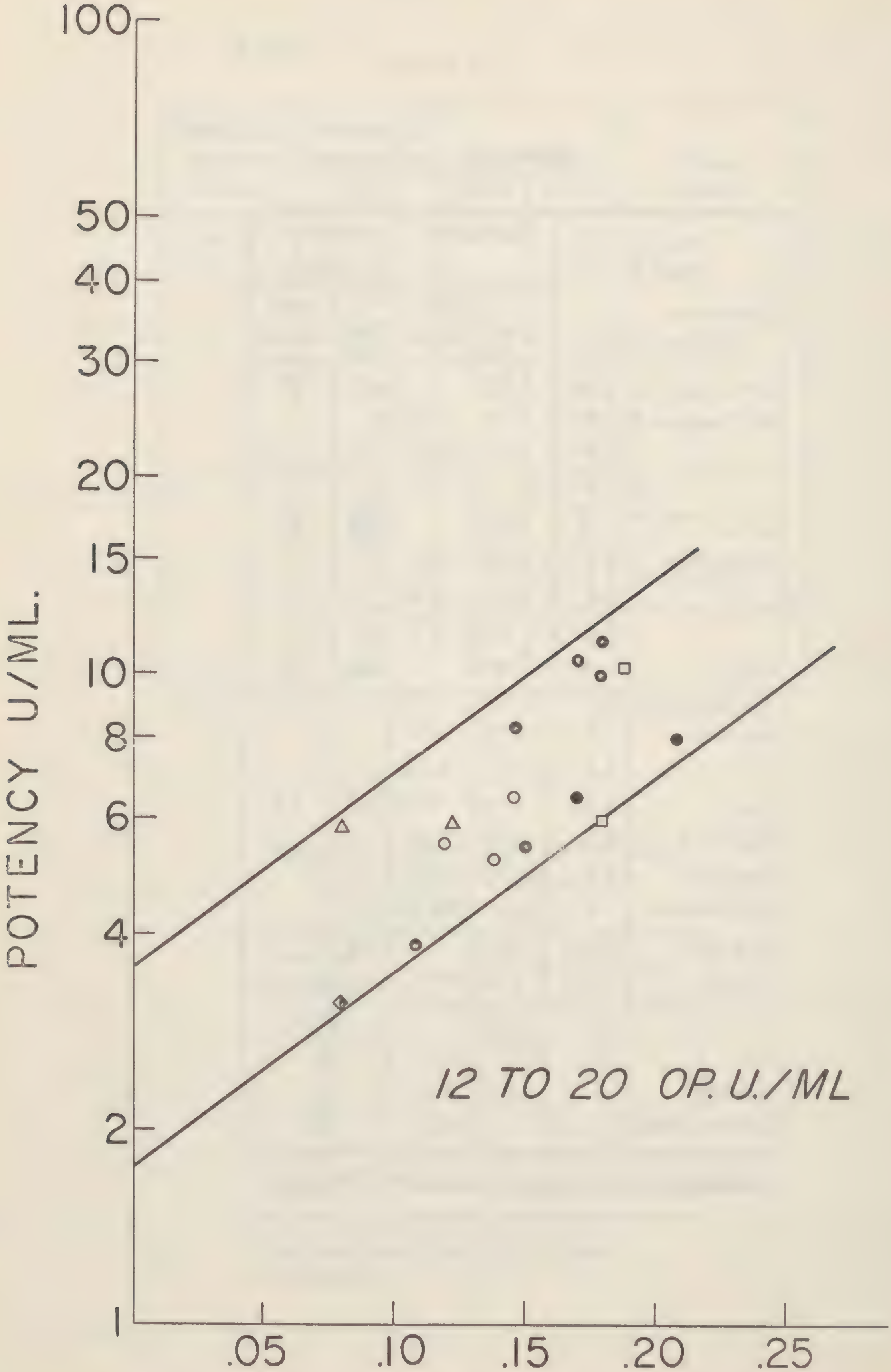
Mice were sensitized with 7.5 op.u. of vaccine.

(Slide 3)

Sensitizing Vaccine	op. u.	Deaths/No. 4°C	Deaths/No. 35°C 48 Hr.	Potency (u/ml) 4°C	Potency (u/ml) 35°C 48 Hr.
Lot 6	1.25	1/10		4.7	3.9
No Pres.	2.5	5/10	6/10		
	5.0	6/10	7/10		
	7.5		7/9		
Lot 6	1.25	2/10		4.6	6.0
Merthiolate	2.5	2/10	2/9		
	5.0	8/10	7/10		
	7.5		8/10		
Lot 6	1.25	1/9		3.8	2.0
Benzethonium	2.5	3/10	1/10		
chloride	5.0	6/10	4/10		
	7.5		6/10		
Lot 6	1.25	3/10		2.5	1.8
Methyl- &	2.5	6/10	2/10		
propyl-p-	5.0	6/10	3/9		
OH-benzoate	7.5		10/10		
U.S. Std	0.625	3/10		8.0	
No. 6	1.25	6/10			
	2.5	8/10			
	5.0	10/10			
Control	–	0/10			
(no vaccine)					

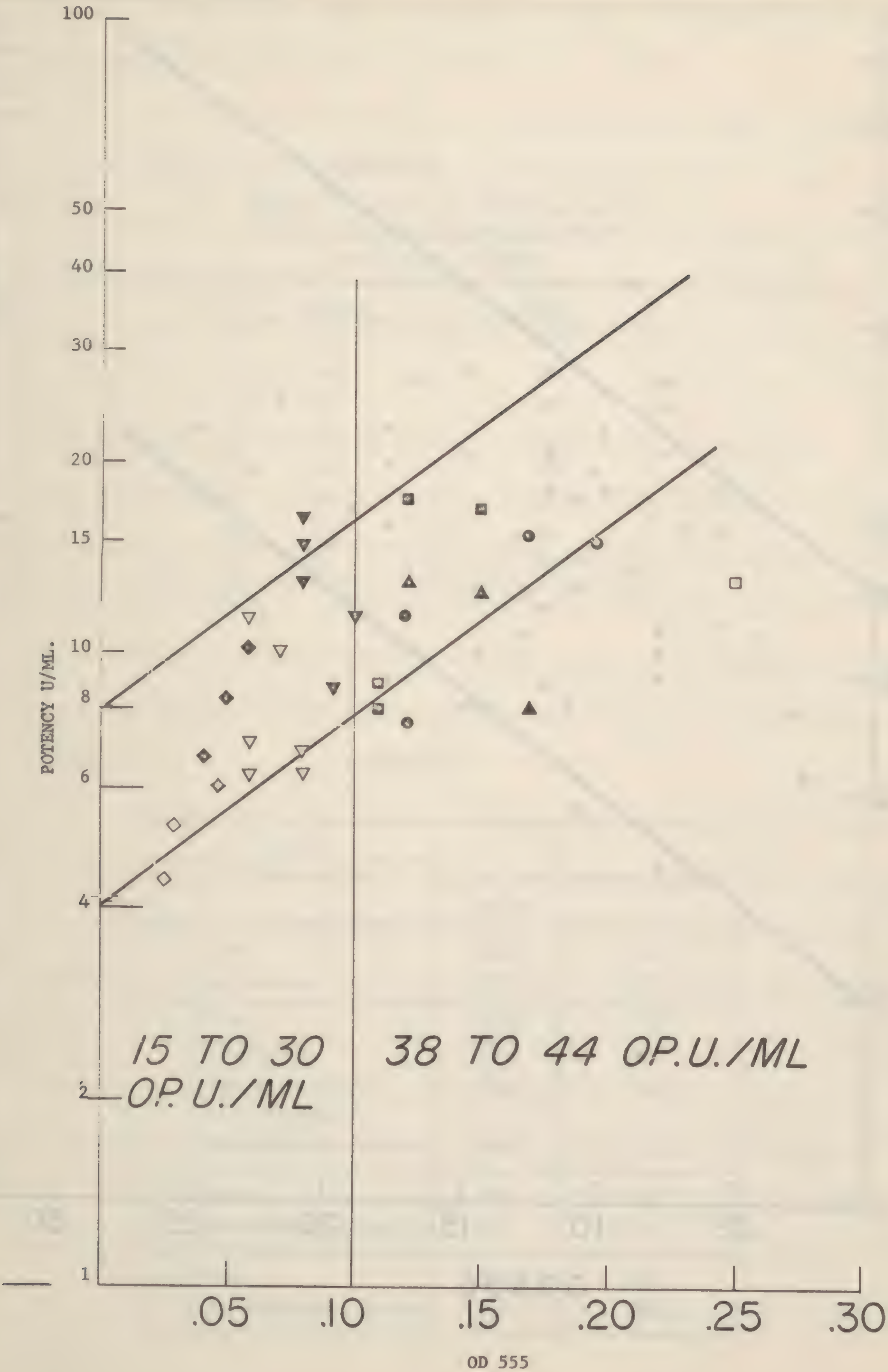
Challenged with histamine diphosphate 100 mg/kg.

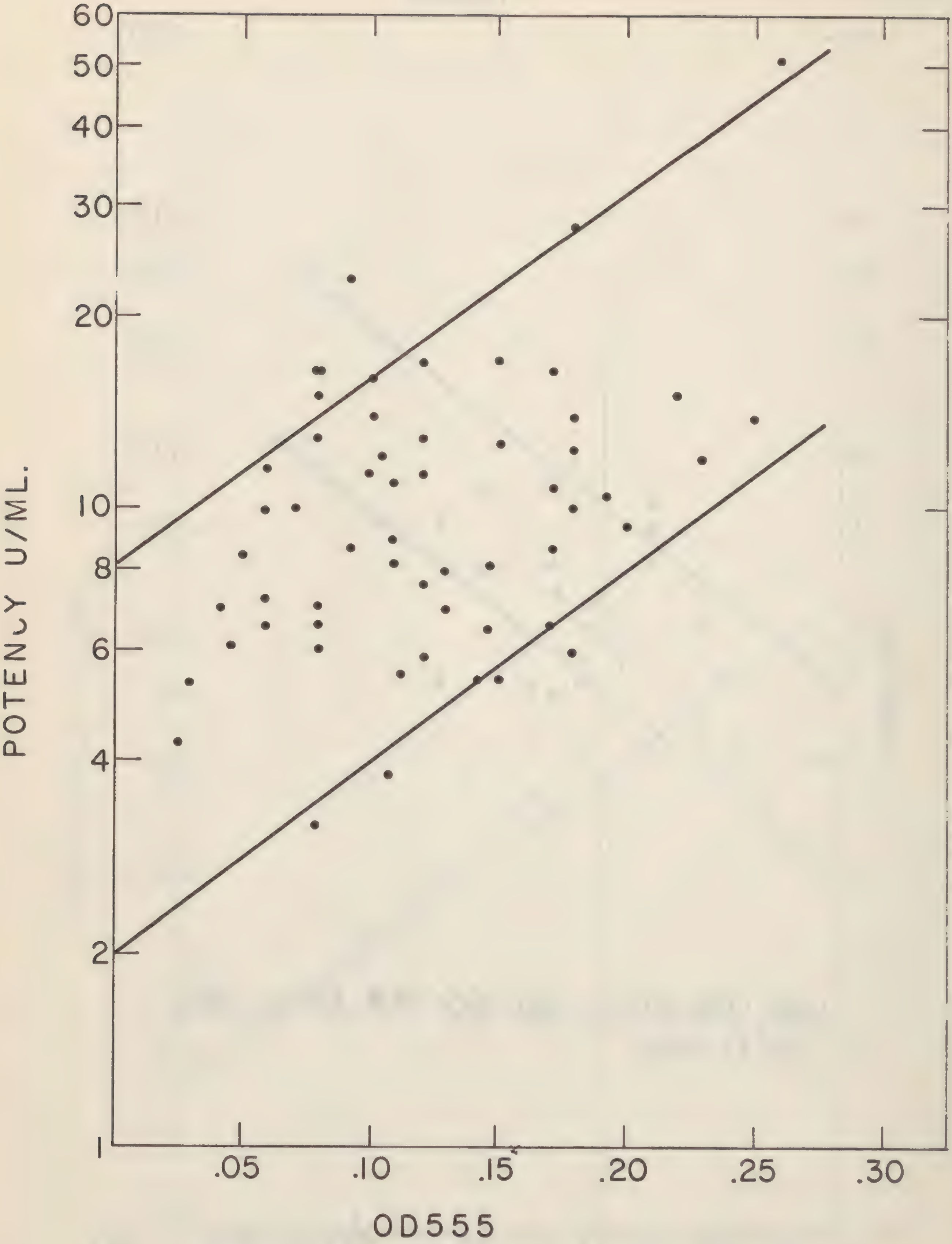
(Slide 4a)



OD 555

(Slide 4b)


100
50
40
30
20
15
10
8
6
4
2
1
POTENCY U/ML.
15 TO 30 OP. U./ML
38 TO 44 OP. U./ML
.05
.10
.15
.20
.25
.30
OD 555


60
50
40
30
20
10
8
6
4
2
1
POTENCY U/ML.
.05
.10
.15
.20
.25
.30
OD555

(Slide 6)

BIURET READING OF SEVERAL LOTS
OF VACCINE BEFORE AND AFTER
30 MINUTES AT 100°C

Before	After
.03	.04
.065	.07
.07	.09
.12	.13
.14	.15
.20	.22
.23	.24
.26	.28

(Slide 7)

Vaccine			
Lot	Preservative	Sialic Acid μ moles	Potency (u/ml.)
1	None	.0045	8.5
	Merthiolate	.0039	12.1
2	None	.0078	8.6
	Merthiolate	.0058	4.4
	Methyl- and propyl-p-OH-benzoate	.0068	3.2
	Benzethonium chloride	.0065	2.5
3	None	.012	Nil
	Benzethonium chloride	.010	Nil
4	None	.011	Nil
	Benzethonium chloride	.011	Nil

and time has gone into the work which was presented today, and I am going to ask Mrs. Cook from the Texas State Department of Health to open the discussion of these papers.

MRS. COOK: This problem of stability in the vaccines, of course, we are all very interested in. Just how long we can hold a pertussis vaccine or store pertussis vaccine and have it remain antigenic under ordinary conditions of storage in our manufacturing plants.

We have heard here this afternoon a number of points that affect stability of vaccines -- the storage temperature, for one, length of storage, effect of the preservatives. But I don't believe we heard much about the type or manner of killing agents, per se. Nor did we hear too much about the type of diluents used, whether buffer, what the pH might be and so on.

We did hear something about the influence of the adjuvants, and Dr. Anderson's work on what happens when we distribute these products in shipping them about the country. And also some indication that other antigenic components in the mixed preparations might influence them.

But aside from these points, what other things might influence stability or how can we say that a vaccine is stable, is potency the only criteria, or must we look for physical changes like aggregation or color changes and so on that might happen to these products?

I think we have a wide open field for discussion, and I hope we have lots of questions.

DR. WILSON: Thank you very much, Mrs. Cook.

Now, the papers grouped themselves into three groups -- one concerned principally with stability, and perhaps we could open the meeting for discussion on this particular group of papers first.

Dr. Barrett.

DR. BARRETT: Mr. Chairman, if I may, I happened to bring along in my pocket, so to speak, some data that I believe would so show that not all the evidence is pointing in the same direction in terms of the responses in infants to pertussis antigen as measured by the agglutinin titers, of course, and particularly, I would like to go up front and ask our projectionist to show the first slide.

Over a period of the past five years or so, we have run numerous trials, and this data was put back in the vintage of about 1959 or '60 before I had any inkling or heard anything about this question of pertussis antigen degradation, loss of potency upon storage. So we were swinging along with vaccines that present day standards wouldn't be used, certainly with any intention unless it was purely for experimental purposes.

The material that we are looking at here is quadrigen, formerly processed by Park-Davis.

In those days, we were using this particular lot, 7513,

in one study number 7. It was five months old from the date of what the manufacturer calls pooling or blending of the components to the time that I first used it as the first injection in a series of four doses, a month apart, in infants one to three months of age.

Then, approximately six months later, we recalled these children, bled them, of course, and gave them a booster, same dose, same material. And we see here that in comparing this study number 7 to another one, number 8 -- again, infants with similar age -- by this time, the vaccine, however, was 12 months old, and in my work, I use percentile distributions rather than geometric means or scattergrams, so that the lower level, for instance,here represents the height of the titers of the lower 10 per cent responders in any given study group. And the upper limit of the gray area represents the heights reached by 90 per cent of the specimens.

The interrupted line represents the median or average level. So that if anything, the material improved with age.

Now, I don't make a case over that at all excepting I say it is an empirical observation that I thought worthy of presenting, and we have several other ways of showing the same thing. That is, different studies showing fragments of data all pointing in this direction that as we can measure the responses in young children as well as children three to six months of age and another group one to three years of age, we couldn't

see any fall-off in agglutinin titers as the vaccine aged.

The next slide, please.

I pulled out a lot of quadrigen which was 12 months of age when it was used in the primary series or a lot of triogen or DPT. The triogen, approximately the same age within a month, different manufacturer, and again there is certainly this drop-off. That is, the potency, the antigenic potency, of the pertussis in the quatrigen is no worse off than it was in this particular batch of triogen.

As a matter of fact, it looks better, but that doesn't prove anything in that sense other than you couldn't see any significant difference.

I hasten to reiterate that these were not controlled studies. It simply, I think, opens up the door to question the problem in the other direction.

The next slide and the final one is a study where the objective was to take the same lot of vaccine at different doses at different spacing between doses for the primary series, and if we can have this shadow removed for the moment here, this is our reference schedule -- four doses a month apart. And in each one of these schedules, you will notice there was an injection given of the same vaccine at the ninth month in terms of time. This, we might call the challenge.

The next schedule was a three-dose schedule, a month apart, three doses two months apart, two doses a month apart,

two doses three months apart, a single dose, primary, if you please, and no primary. And this would, of course, be our control.

Here, the upper 10 percentile, and we are looking only at the pertussis, these were specimens run out for tetanus and diphtheria. And the other slide, we have it for three types of polio. But just look at the pertussis and the upper limit of the dark area is the 9 and 10 percentile response. But more interesting to me is the 10 percentile because the higher it goes, the more interesting it is in toto.

The reference schedule four months apart gave best response. Slightly lesser, but hardly appreciably significantly different were three doses -- just a moment; I better begin again.

When the three doses are given two months apart and even one dose, that does sensitize the children or condition them immunologically even though there is no response in the median titer at all after the same time point in a primary series.

The other children were bled when given a second dose nine months later. They boost, you might say, from nothing detectable to a very respectable level, certainly far superior to the reference control which shows no response.

This dark section going up here (indicating) represents only 10 per cent of the specimens which went up. In other words, at the 9 percentile.

Thank you.

DR. WILSON: Thank you very much, Dr. Barrett.

Is there any -- Dr. Edsall.

DR. EDSALL: May I, too, show a slide, Mr. Chairman?

DR. WILSON: These are quite unpremeditated, ladies and gentlemen.

(Laughter.)

DR. EDSALL: We have been trying to solve some of the problems of the mechanism of the instability of pertussis vaccine in the poliomyelitis combination situation. And when I say 'we', I am speaking of ~~Mr.~~ Miss Wetterlow who is at the other end of the table and myself. And I think I am mostly the cheering section.

We have put some effort into trying to determine the interaction of preservative and heat with some secondary interest in time and in adjuvants.

The entire work we have done could be summed up in this piece of paper (indicating), which I told Dr. Eldering our photostatic apparatus isn't that big.

I left a sheet like that on the table in Prague a year ago last June. It promptly vanished out of the dining room. I never could find it again, and I don't know whether they have decoded it yet.

(Laughter.)

To make things a little simpler, I have on the slide

here a few key points that I think have been of interest to us.

In the fall of 1960, we made up a batch of pertussis vaccine which was held for several months to detoxify. And in order to get it off to a uniform start, it was then split into aliquot, treated with either merthiolate or Phemerol, merthiolate and then heated, or Phemerol and then heated, -- I will go back to these later; forget them for a moment -- or in reverse, heated and then merthiolate or heated and then Phemerol.

The unit potencies are shown for the tests that we have been able to run. I won't put much accent on time interval because the major interest that we had here was the comparison of these pairs.

The merthiolate-treated material, although it had a gradual fall and the usual distracting leap from one side to the other of the bell curve of the tests, ran on the order of, say, the potency of six, the Phemerol-treated a good deal lower.

"NC" means non-calculable.

If you heated afterward, it fell off, but the Phemerol preparation drastically more as far as we can make out.

If you heated first and then added the preservative, the fall in potency appears to be relatively minor, and it doesn't seem to matter here whether you add merthiolate or Phemerol, in contrast to the very striking difference that appears to have shown up when the preservative was added first.

Dr. Edsall

EFFECT OF PRESERVATIVE, HEAT AND ALUM

Year	Treatment of Vaccine			
	M	Ph	M & H	Ph & H
1961	7, 7.2	2.7, 3.8	2.4, 4.6	NC, NC
1962	9.2, 3.4	NC, 1.3	6.0, 1.7	NC, 1.7
	5.1	NC	4.3	
	DPT-P	DPT-P	H, M	H, Ph
1961	4.8, 14.4	4.6, 4.5	5.5, 2.9	4.4, 3.3
1962	6.7, 6.6	NC, NC	2.6, 3.8	NC, 2.8
	NC	1.6	1.0	2.1
1963	NC			

Just to find out what happened when you made up a DPT-polio, we are grateful to ~~Morris Hilliman~~ Maurice Hilleman for supplying us with some surplus polio vaccine a couple of years ago and centrifuging the pertussis, adding the diphtheria and tetanus, suspending it in the polio vaccine without an adjuvant. It was really not worth measuring, but with an adjuvant, fairly good potency, again, the material derived originally from the merthiolate-treated, but some Phemerol was now present, and you see over a period of time a fall in the potency as you might have predicted.

This is all stored in the refrigerator. This Phemerol-treated preparation, the original pertussis, when ~~transmogrified~~ transmodified this way into a DPT-polio preparation is, if anything, perhaps somewhat lower in potency and falls off faster as far as one can tell from the limited number of tests that we have been able to carry out.

Even against the distracting variations in the nature of the tests, it is our impression that there is a significant interaction between heat, preservative and sequence of treatment here. We don't know yet what it is, but we do feel it worthwhile to present these findings as an indication of a direction in which perhaps a little more careful study may yield more useful information.

Thank you, Mr. Chairman.

DR. WILSON: Thank you, Dr. Edsall.

Yes, sir.

DR. DEVLIN: I hate to belabor this point of Phemerol, but I would like to make some comments on behalf of the work we are doing at Park-Davis.

We have gone to our Statistical Methods Department, and they have drafted a series of eight experimental designs in connection with quadrigen which contains Phemerol. These have run the whole gamut of variables, shaking temperature, container and all the components separated.

The reason for doing this, of course, is we could not make up our minds why certain lots of quadrigen appeared to be stable and others weren't. And we had shipped material to DBS, and they would find low potency. And when it came back, we would find the same thing.

Our studies to date -- they are not completed, and we may have some drastic changes in them now that the Michigan Department of Health has conducted such beautiful studies -- but we do find that shaking has an influence on the pertussis, and this is without preservative.

It seems that when you do decrease the potency that Phemerol does not seem to add to the deterioration. It would appear as though the polio in some way offsets the effect of shaking. This is polio and pertussis, no preservative.

The phosphate product holds up well, just phosphate and pertussis. If you put polio with Phemerol, again, you

offset some of the deterioration, but not all of it.

I just want to add that we were next going into a study of the phosphate system. As you know, we also have some protamine in quadrigen so this is a factor that would have to be studied.

But unpreserved, when we studied a freshly made preparation of quadrigen, except with and without Phemerol, definitely the unpreserved material held up better. And we did accelerated tests. All sequences are done in the mouse-protection test, and we accelerated by shaking either at plus 4, room temperature, and 37 degrees.

I think that the answer to the instability of some lots might be the timing on getting a phosphate into the product now and, of course, might involve the presence of unknown cations.

DR. WILSON: Thank you very much.

Are there any further comments on stability?

Dr. Perkins, you have recently acquired a DPT-polio in Britain. Have you any comments to make with regard to stability?

DR. PERKINS: No, except that the manufacturer concerned did all the tests for stability of the pertussis component and, secondly, it maintains its stability for at least one year.

They have run into all the problems that you ran into earlier in this country of merthiolate deteriorating the polio component. But the pertussis component stability has never been

a problem with them.

I don't know whether Dr. Fantes would like to add anything to that.

DR. FANTES: Well, I would like to add we use no preservative whatsoever in our quadrigen. We use, however, a low level of versene, EDTA. Whether that has any effect or not, we don't know.

That was partly prompted by previous work by Dr. Corkill with which we were familiar where he thought that EDTA had a beneficial effect on the stability of the pertussis component. But as Dr. Perkins said, our tests have not been able to show any deterioration of the pertussis component in our quadrigen.

DR. WILSON: It seems to me that Glaxo has been using a purified poliomyelitis vaccine. Have you any comment to make on that?

DR. FANTES: Well, certainly, our earlier batches consisted of purified material. By "purified", I mean material whether it is calcium phosphate and reduced, which eliminates 90 per cent of the extraneous protein.

Now, at one time we thought that proteolytic enzymes which are sometimes found in finished polio vaccines could have an effect on the pertussis stability. Certainly, the element in phosphate treatment reduces or eliminates the proteins quite appreciably. And we attributed perhaps the stability of our

pertussis in our quadrigen to the absence of proteolytic enzymes. But, again, we don't know whether that is or not.

We certainly found when we used deliberately high protease-containing material -- that is, we added trypsin -- the pertussis component did deteriorate more rapidly than in normal materials. But whether the naturally occurring protease in polio vaccine has any part to play in any reduction of stability, I wouldn't know.

DR. WILSON: Thank you.

Yes, sir.

MR. SCHUCHARDT: I would like to relate a couple of our experiences that we have had with our product Tetravax which follows closely what the British people evidently are experiencing.

When this product first came out, the company was interested in polio vaccine, and the unfortunate Cutter incident came along and kind of set us back for a while in timing. But we had made this vaccine, and the material was stored around the cold vault for a period of a year, some of it two years.

And it was this first material that we were given to prepare our final Tetravax samples with. And I guess when we got it, it was at least a year and one-half, two years old.

We did our initial stability studies on our first lots which was made from this material, and these looked very good. We got up to 36 months' stability on some of them.

Well, the first lots that hit the market came out, of

course, for the older polio, but as you sell this off and the new material comes through the pipe line, we started making our ~~tetravacs~~ Tetravax with a new material, and this is when the people in Boston found that lo! and behold! the material was not stable.

And we checked this, and we found it wasn't stable. And Dr. Pittman did. And this created quite a fiasco around the company, of course.

Dr. Pittman at this time suggested the enzymatic theory about this, and everybody knows the work of Baron here who reported the ~~protolytic~~ proteolytic enzymes in polio vaccines. And here,we thought, was a case where we could do this.

We took polio vaccines which had been in our cold vault, samples of these, for a number of years, and some were fresh materials. And simply by adding skimmed milk to this, we could demonstrate we did have protolytic enzymes in the newer material. And we had a hard time finding any enzymatic activity in the old material.

Now, we combined this material and made pertussis vaccine. And I wish I could tell you we got results, but, unfortunately, at the time they were going potency, they dropped interest in this, and we never tested them. So I don't know how this would have come out.

But I often wonder if anybody else had worked along these same lines, and I am glad to hear that the Glaxo people have done something on this.

DR. WILSON: Thank you very much, Mr. Schuchardt.

Are there any other comments?

Dr. Pittman.

DR. PITTMAN: I might just add something to Mr. Schuchardt.

We carried on some of his vaccines that were prepared with the ~~tetravax~~ Purivax, and we got no loss in potency with that.

MR. SCHUCHARDT: Of course, this was the answer that we had.

DR. PITTMAN: Yes.

I would like to ask Dr. Barrett, in presenting his agglutinin titers, you were comparing quadruple antigen with triple, but with the quadruple, you were giving four doses and a booster.

With the triple, how many injections?

DR. BARRETT: Four doses.

DR. PITTMAN: They got four with the triples?

DR. BARRETT: Yes.

DR. PITTMAN: It would be interesting to know what were the protective activities of the sera of these people. You have the high agglutinins, but does this correspond with protective activity?

We do know that if you took the early preparations of agglutinogen -- in fact, they used to do skin tests -- and one or two doses, and you would get a very high agglutinin

titer in these children. But if you tried to do protection tests with the agglutinogen, this wasn't anything.

So I am just wondering if this was agglutinogen reaction titer, but not protective titer.

DR. BARRETT: Dr. Pittman, I am betraying my ignorance here. How do we go about measuring protective titer in the ~~mouth~~ mouse?

DR. PITTMAN: I wish I knew, Dr. Barrett. I certainly wish we had a measurement.

DR. BARRETT: I wondered if you were saying that we did have something that I hadn't been aware of.

All I can say is that there are straight agglutination titers standard techniques. ~~Park~~ Parke-Davis Laboratory performs them, and their people are here and perhaps Dr. Timm might want to comment on that.

Again, this is nothing controlled, but I have kept up a monitoring -- controlled to this extent -- of every child that we have ever had in a trial that has involved the use of the DTPP quadruple preparations, any of the combined vaccines containing certain pertussis-polio. The monitoring has amounted to matching every case of reported whooping cough that comes to the attention of our Epidemiology Section of the department against every child at any study that I have conducted who had received at least three doses of any one of these variety of preparations. And of some 2500 children that have

been so involved to date, there have been two cases of whooping cough reported.

These are not bacteriologically confirmed.

DR. ELDERING: Mr. Chairman.

DR. WILSON: Dr. Eldering.

DR. ELDERING: I might say we still have some of these vaccines, some with Phemerol and the cations and so on and some from incubator and some of the icebox. And we are starting to do some agglutinin production tests with these vaccines in mice, and we hope to find out whether these deteriorated antigens will produce agglutinins.

DR. WILSON: Dr. Edsall.

DR. EDSALL: Mr. Chairman, I wonder since we are fortunate to have an expert on the relations of agglutinins and protection here sitting right behind me, we couldn't persuade Dr. Perkins to comment on what, if any, relationship he thinks there might be between them.

DR. PERKINS: You know you are talking to an interested party here.

I firmly believe that a good vaccine will produce good agglutinins. And the correlation we got, as you know, between the vaccines that gave good protection in the field and production of agglutinins in mice was extremely good and a better correlation than that we got from the mouse-protection tests.

And the only vaccine that let us down, as it were, was Pillemer's antigen. And, of course, I was using the wrong strain to do my agglutinin tests with ~~four~~ for Pillemer's antigen, which is a most unusual strain as far as Bordetella pertussis is concerned. And I would certainly like to reassess this.

I think, however, we ought to be extremely careful of the strain we use for doing our agglutinin test. This, I think, is coming up now in this work of Preston where we see there are antigenic differences in the strains that are being isolated from whooping cough cases. And this will have a bearing upon our agglutinin tests.

I am afraid I can't say any more about the correlation between agglutinin production and protection.

DR. ELDERING: Isn't it possible that every good ~~wholesale~~ whole cell vaccine may produce agglutinins, stimulate the production of agglutinins, but everything that stimulates the production of agglutinins isn't a good vaccine. Isn't that true?

MR. SCHUCHARDT: I am afraid not, Dr. Eldering. We got burned on this.

We went out and did a chemical study on material made with pertussis which was excellent protective antigen producers but unfortunately, it didn't produce agglutinins. It had very, very little agglutinin in it.

DR. ELDERING: I should have said, "most of them."

MR. SCHUCHARDT: We had to look for pertussis antibody.

We were really hard pressed.

DR. ELDERING: Did you use the right strain?

MR. SCHUCHARDT: Yes, we tried homologous strain, and it didn't work. And we ended up going to a high agglutinin test.

DR. WILSON: Are there any more questions or comments as regards the stability of the pertussis component for any reason?

Dr. Corkill, it seems to me you did some work at one time that may have a bearing on Schuchardt's experience with the addition of polio to pertussis. Where it affected, didn't it affect turbidity pretty markedly?

DR. CORKILL: At one time, we had various preparations of poliomyelitis vaccine which we could definitely show or, at least, we definitely showed that there were perhaps two fractions in the poliomyelitis vaccine -- one which lowered the turbidity of the pertussis suspension, and the other which affected an antigenicity of the suspended pertussis.

Actually, we are able to show that pertussis was stable in the poliomyelitis medium, but in washings removed from growing kidney cells before infection with the poliomyelitis organisms, these washings also affected the turbidity and the antigenicity of pertussis vaccine suspended in them.

I don't know what change happened. We have had subsequent lots of poliomyelitis vaccine from our Polio

Department. And of these, some vaccines have been very dramatic in lowering the antigenicity of pertussis when the polio-pertussis mixture was incubated at 37 degrees for about ten days.

Subsequent lots of polio or recent lots of polio have been rather disappointing in this respect and some of the earlier work also showed that perhaps this effect of the poliomyelitis vaccine was removed by purification process perhaps as described by Dr. Fantes of Glaxo.

Recently, we have been trying to repeat some of this work, using purified polio and crude polio. Unfortunately, our crude materials have not been very active in destroying the pertussis antigen. So some of our work to purify material has not been successful.

DR. WILSON: Thank you, Dr. Corkill.

Dr. Fantes.

DR. FANTES: I would just like to add that tissue culture from monkey kidney cells are very rich cells of ~~protelytic~~ proteolytic enzymes, but the mere filtration processes involved in a production of a polio vaccine remove most of this ~~protolytic~~ proteolytic activity. So the fact that the crude material is detrimental to pertussis stability while the finished product is not could perhaps be due to the ~~protolytic~~ proteolytic enzymes.

DR. WILSON: Thank you, Dr. Fantes.

Yes, Dr. Anderson.

DR. ANDERSON: We have run a number of ~~protolytic~~ proteolytic

tests on the polio component which we received. We buy our polio component, and it is prepared by a Maitland process which means simply macerating the kidney tissues, I understand, instead of trypsinizing. And we have yet to detect proleolysis in the polio component. So we have pretty much run it off.

DR. CORKILL: Mr. Chairman, I might mention that if merthiolate is added to these polio preparations, then, if you suspend your pertussis in them, the merthiolate polio or merthiolate washings from the polio, then the pertussis is quite stable.

DR. WILSON: Thank you.

DR. TIMM: I would like to mention when the question was first raised, there was this problem that Dr. Corkill raised of the effect of polio or enzymes from such products. We did put on an experiment to test, not only effective final polio lot, but the effect of the various components that would arise during the process of production of polio vaccine. We did check the effects of the original harvest from the monkey kidney bottle cultures prior to infection with polio.

We checked the non-infected harvest and the infected harvest prior to coarse, medium, fine, and ultrafine filtration and after filtration. We checked the same thing after from infected cultures. We checked the same thing as a final product--that is, after the additional aluminum phosphate, all of these with pertussis for a period of 28 days at 37 degrees.

In one single experiment, we could tell no difference in the results, and that is as far as pertussis potency is concerned.

I would like to raise the question relative to the agglutinin response that Dr. Barrett talked about. The question, of course, is one of practicality when you are talking about large-scale clinical studies to a large extent.

However, when we started running agglutinin titers to detect response in humans, it was based on the assumption and partly on the results that had been shown by the British studies that there was pretty good correlation.

And I have a question at this point of Dr. Kendrick.

I have got the impression from something that you mentioned as well as some other points that were brought up during the meetings yesterday that you felt that there was a good correlation and that where there wasn't, you usually found agglutinin titers in the absence of protection titers. Is this a correct interpretation or not?

DR. KENDRICK: Well, we discussed tests in animals rather than in children for the most part. But we have quite a lot of data in children.

And I believe I did make the statement that if the antigen that was being used for a vaccine was whole cell antigen, you expect a response in terms of agglutinin. If, however, it is possible to fractionate the antigen in such a

way that you have a protective component that is free of ~~agglutinin~~ agglutinogen, how can you expect to use an agglutinin as a measurement?

Now, I think Mr. Schuchardt has demonstrated, has he not, the isolation of ~~agglutinin~~ agglutinogen again which would not protect. Would he want to confirm that?

MR. SCHUCHARDT: Well, it isn't immunology.

DR. KENDRICK: Yes.

I think the antigens we are talking about today are whole cell antigens, and you would expect an agglutinin response. However, that is different than saying that the agglutinin per se is the protective thing.

DR. TIMM: That was my point. I was talking about whole cell.

DR. KENDRICK: That's right.

DR. WILSON: Thank you, Dr. Kendrick.

Are there any more questions or comments concerning stability?

If not, I think that we should pass on, then, to the subject of rapid assays for detecting loss of potency.

Has anyone any questions or comments to make with regard to Mrs. Gardner's paper?

Mr. Marshall.

MR. MARSHALL: I would just like to ask: Is the relationship of the histamine-sensitizing factor to potency to

the mouse antigenic factor constant, or is this a casual relationship such as you find with LF and the antigenic components of toxoid?

MRS. GARDNER: All I can say is it is constant in those I have tested.

DR. DEVLIN: I think I wanted to ask this question of Dr. Munoz, but I think due to Dr. Munoz' paper, we have to be a little careful that possibly the groupings on the protective antigens, certain groups permit HSF activity and other groups protect.

?

But the Mewar article indicates that HSF is not as stable as the protective activity. So, of course, if you are going to use HSF for screening, you would have to be careful that you keep it under carefully controlled conditions.

DR. WILSON: Thank you.

Are there any more questions or comments?

Yes, Dr. Munoz.

DR. MUNOZ: I think it would be a mistake to substitute the histamine-sensitizing factor tests for the protective test at this particular period of development. We have indications that some treatments may affect the histamine-sensitizing factor differently than the protective activity. And I think that this point is well taken by Dr. Devlin.

So at present, I would hesitate to recommend anybody to substitute this test, the protective test. But for screening

test, I think at NIH, the results represented by the group are very clear and certainly they accelerate any program.

And as I told you, our work was done originally all on HSF, and it was not until we had this reasonably pure form that we started to test for protective activity. And we found at that time that they both correlated very nicely. But one should be cautious to say that they both will always correlate because they most likely will not.

DR. WILSON: Thank you, Dr. Munoz.

Are there any further questions?

If there are no further questions, ladies and gentlemen, I would like to thank you, and I will turn the meeting back to Dr. Pittman.

DR. PITTMAN: Well, we do have a little time left, and there were several questions that we wanted to bring up.

Would you like to discuss about strains? What are the quality of strains that make good antigens?

Another one was what should be the size of the booster dose? Must we give a whole single injection or can we cut this down to one-half or even less of the single immunizing dose?

There was a third. What was the third one we wanted to discuss?

I heard "strain of mice." I think the mice have had it.

(Laughter.)

But we have a few minutes we might use profitably. Someone has proposed that we get a collection of good strains, study them from various standpoints. Do you think this would be a profitable thing to do? Shall we look for strains that are free from toxicity? Would this help in making more acceptable vaccines?

Do you want to discuss this for a few minutes? Who has some recommendations?

DR. KENDRICK: Dr. Pittman, would there be any lots that were particularly free of toxicity and that you could start with the strains that were used for that lot and test those strains?

DR. PITTMAN: I think that this would have to come from a manufacturer because by the time we get them, I don't know what kind of treatment they have had.

(Laughter.)

DR. KENDRICK: I think this question is one that could be talked about by the manufacturers. Do they have lots that are particularly free of toxicity?

DR. MILLMAN: I think we could expand that, Dr. Pittman, to include growth conditions, and I think Dr. Cohen might even expand this with some of his studies that I heard last year dealing with continuous flow cultures where he has actually shown the separation.

DR. COHEN: What we actually did do is we have this

pertussis organism in continuous cultures, and we varied the dilution rate of the culture, feeding rate, between 0.04, and I think, 0.12. It means that every hour, about 10 per cent of the culture is exchanged.

We found, then, starting with a very old culture, about 18 hours old, it hadn't any protection at that moment. And by varying the dilution rate further at a certain moment, we got back protection. And when we added a considerable amount of medium again, the protection got lost again. And this ran parallel with sensitizing factor, but not antigenic factor.

What we are trying to do now at this moment is to have these cultures at a constant dilution rate, but putting up, let's say, four cultures with different dilution rates parallel with each other. And now we don't find by doing it that way -- this was the first experiment with changing the culture, changing the dilution rate, renewing the culture process in the same culture.

Now, we are trying to get other dilution rates applied to cultures in parallel. And you find there is no mention of protective antigen which can be obtained. There is a certain moment that the culture grows too old because we don't give any new and fresh medium in it. And there is another moment in which the culture is too young. We wash out the bacteria by adding too much medium. But between both, we can get a fairly constant amount of protective antigen and histamine-sensitizing

factor.

We do not know yet about the toxin and how it is formed, but it can be studied with such a model. I agree with you in this respect.

DR. WILSON: Dr. Edsall.

DR. EDSALL: Did you want to reply to Dr. Cohen? Excuse me, I wanted to raise a question.

DR. PITTMAN: No.

DR. EDSALL: It seems to me at the Prague meeting a year ago last June, fairly good evidence was brought out of a negative sort that showed a lack of relationship between established toxicity tests between animals or in the laboratory and toxicity in man. And I haven't heard that there has been any basic change in that state of information.

So just to be Devil's advocate, I would like to ask why we do toxicity tests.

(Laughter.)

DR. PITTMAN: You should have been here yesterday.

DR. WILSON: We will send you the transcript of yesterday.

(Laughter.)

DR. PITTMAN: Dr. Devlin?

DR. DEVLIN: Concerning strains, you heard people say that a prolific growing strain is not a good strain for a vaccine. I believe it is stated that we should use mixed strains,

isolates, at least to prepare a vaccine.

We did obtain the strain from Billaudelle and through Malmgren, and it grew very well, had low toxicity, as a vaccine had pure potency.

I just feel that there could be some type of work done on predetermining possibly as to what strains to use. I think that our strains are old. They have been stored for long lengths of time in the live state, but as to whether the new isolates ought not to be obtained. I am speaking from ignorance.

DR. ANDERSON: We had some data a number of years ago where we were adapting the fermentation methods to the production of pertussis where we could produce tremendous quantities of vaccine with no potency, using the same seed. And it resolved itself at least at that time for our working operations to very carefully plating out the seed and selecting colonies through separate flasks and fermentation. And then we got potency.

I hope that helped, but we did find the ones that were tending to become atypical, overgrew the antigenic strain in the submerged culture where you don't see this on BG.

DR. PITTMAN: Was there any difference in the toxicity of those?

DR. ANDERSON: I don't remember.

Do you remember? You did the testing on that.

DR. ELDERING: No, I don't remember.

DR. PITTMAN: I have a recollection that Dr. Else ~~Elsie~~ Krag Andersen ~~Craig Anderson~~ has a strain she called atoxic. Has anyone ever used that?

DR. ELDERING: That's a New York strain she got.

DR. PITTMAN: Is that a good protective antigen?

DR. ELDERING: We have tested it before it was atoxic. I don't know what that means.

(Laughter.)

DR. DANIELSON: Dr. Pittman, what do you mean by atoxic in mice?

DR. PITTMAN: Yes.

DR. DANIELSON: That is what was worrying me. I think when you mentioned here from the manufacturers, if you have an extra good batch atoxic, what do we mean in mice, again?

If we mean this experience in the field, I don't know how the others are, but I wouldn't have much of a chance to find out from my own company. The only time I hear of anything is when it is really bad.

If you are talking about toxicity in man, at least I won't have a way of knowing if we have an extra good batch.

DR. ELDERING: We are still talking about mice, I am afraid.

DR. PITTMAN: Surely, strains do vary in the degree

of toxicity. This is just normal to expect that they are different. In all bacteria, we have different relative amounts, components of different constituents.

Dr. Barrett.

DR. BARRETT: Dr. Pittman, I would like to talk about this reduced booster question if you are ready. I would certainly like to see and would like to be a part of any trials that would evaluate this question.

I haven't heard anything that says we are ready to accept the reduced booster in principle as yet in terms of a preschool youngster. But I do want to point out from my own experience that you have got to be careful. What we are talking about, of course, what is a booster.

The younger we start in infancy with the immunization schedule, the more critical this becomes. And with the pressures, the desires, of practicing pediatricia , general practitioners, to get started at two months of age or sooner if they could, they may be doing, but the Academy of Pediatrics, having a schedule as we all know of two, three, four months for their primary.

In Michigan, we recommend a three, four, five month age. Not much difference, but the important thing is here a fourth dose is given at the age of one year or some seven to twelve months later. I don't consider that a booster.

Borrowing a term or expression from Dr. Edsall's

writings, I would consider that a reinforcing dose or really the completion of the primary series. Whether we give that ~~fox the~~ fourth dose a month later, two months later, six, seven, ten months later after the third, it is crucial that it be given and it be given in full dosage. After that, I would hope that we could speak in terms or work in terms of a reduced dosage.

I would like to see this problem of reactivity in the children minimized to its lowest complaint rate, if possible, and I am talking about now, the fifth dose that is given -- of course, I am talking about Michigan traditions -- at about the age of two or three years. That's when the next dose is given. And then finally, at the age of 4 to 5 years just before they go to school.

In that sense, I would like to see a reduced booster. I think that the material that Dr. Anderson, our colleague from the State Health Department, put on the screen today was somewhat of a bombshell to me. I hadn't seen that part of your data yet.

DR. ANDERSON: That's hot. That's fresh.

DR. BARRETT: That the geometric means were about half of the post-primaries. All other work, we have done. This has been not so. It has been quite reversed.

This is not to argue that point. It is a completely valid observation. But I think that we have got to more than ever look at this question of booster and titrate all the way from a tenth up to a 1 cc level or corresponding unit in terms

of each concentration of the material.

DR. ANDERSON: One of the problems that concerns us mostly right now is the fact that making a quadri-valent and theoretically having it available information for widespread use. We are afraid to reduce the dosage recommendation for this booster mostly because of the polio component.

Until we can get a concentrated polio, I would be afraid to reduce this dosage recommendation. So what I have done on the side, and I don't know as it has official approval from the department yet, after their reinforcing dose, I would much prefer that they go back to DPT where they can reduce the dose nicely and give a separate polio in full strength.

DR. WILSON: I think that what Dr. Barrett has proposed is very interesting, but I think there is something inherently dangerous in using only the agglutinin response to determine the effectiveness of a booster dose. I think the information would be useful, but I think until we have some test whereby we could better assess the protection that is conferred, it might be dangerous.

DR. EDSALL: May I ask Dr. Wilson if anybody has any information on a dose response curve of any of the measurable antigen-antibody system of pertussis vaccine along the lines of what Dr. Barrett suggested might be done? There is a good deal of that type of information on a number of other antigens, but I do not recall that it has been done with any system using

pertussis vaccine.

DR. WILSON: None that I know of, Dr. Edsall, and I think for obvious reasons. I think people would be hesitant to just use an agglutinin titer as an index of success or failure.

DR. BARRETT: Mr. Chairman, of course, I was speaking in terms of trial and not recommending as a means of just going ahead arbitrarily and doing this.

I am pleased to have you point out that admonishment. We must watch our protection levels as well.

DR. PITTMAN: In the meantime, I have thought of the third topic that was on the program. That is the factors that influence potency testing.

This was one subject that several manufacturers asked that this be put on the program, but no one volunteered.

Does anyone want to volunteer now to talk about it? You are all satisfied with the potency test?

You certainly have been very nice. Someone handed me a note. I heard someone say they had never attended a meeting so well timed.

(Applause.)

I want to thank all that you have so graciously stayed within the time. You had your papers well prepared, well presented.

Is there anything further that we need discuss? Do

you want to stay here and discuss further or do you want to be recessed early this afternoon?

DR. MURRAY: I would like to say just a word before we close this afternoon about tomorrow morning's session. This is merely a general discussion about possible requirements that may be adopted eventually as regulations for pertussis vaccine.

I would like to point out that this discussion tomorrow morning is mainly for the benefit of those who really wish to discuss the substance of a document which was circulated earlier to those proposals. And this will not in any way substitute for the formal methods that we have to go through in the adoption of regulations such as preliminary publication and so forth.

Some of you may not even have any interest in such a session, and I just mention it at this time.

DR. PITTMAN: Well, anything further?

If not, then you are dismissed until tomorrow morning.

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